NUMBER 4

OLOGY

THERMOPHILIC BACTERIA

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Received for publication, March 13, 1919

True thermophilic bacteria are those that grow at temperatures above the maximum temperature for the great majority of bacteria, especially the pathogenic forms. The maximum temperature for the pathogenic bacteria is about 45°C. Their optimum temperature is about 37.5°C. The true thermophiles show no growth, or only very feeble growth, below 40 to 45°C. Their development requires temperatures above 50°C., and some are able to develop at a temperature of 80°C., though most abundant growth is shown at 60 to 70°C.

A group of facultative thermophilic bacteria has been discovered which develop at room temperature, about 20°C., and have their optimum temperature at about 50°C., and their maximum temperature at about 60°C. In this group belong some of the well-known spore-forming soil organisms.

Two distinct types of true thermophilic bacteria have been found in the laboratory, viz., spore-forming and non-spore-forming rods, and thread forms belonging in the class of *Nocardia*.

Most of the thermophilic bacteria studied belonged to the spore-forming class and these varied in size of rods, size and location of the spores, and to a less degree in their biological characters.

The sources from which these bacteria are obtained indicate that this group is widely distributed in nature. They were found in the dust collected on apparatus in the laboratory, on clothes, in soil, in sewage, in the gastro-intestinal contents and feces of rabbits and guinea-pigs, on grain and in horse manure.

The isolation of these bacteria from the different materials is an easy matter, using the plate method with nutrient agar medium, the plates being incubated at a temperature above 50°C. Under these conditions the colonies developing on the agar medium are certain to be thermophilic and can be obtained in pure culture by transplanting on to tubes of slanted agar.

The biological characters of the 60 cultures studied showed marked variation, especially with regard to their action on gelatin, milk, starch solution and nitrate solution. Eleven cultures failed to show any liquefaction of gelatin others showed only slight liquefaction, while others showed complete liquefaction in three or four days. The action on the gelatin was ascertained by cultivating at 51° for three or four days, and then placing the tubes at a low temperature to note whether liquefaction had occurred.

Most of the true thermophilic cultures showed only slight acidity in litmus milk, though several showed distinct acidity with coagulation but no digestion of the casein. Most of the facultative thermophilic cultures showed a primary acidity with coagulation followed by a slightly alkaline reaction and digestion of the casein.

The action on starch varied in degree, as nearly all cultures appeared to have some action in splitting starch. A number of the cultures changed the starch to dextrin, maltose and glucose, though in many cultures no other change than the dextrin stage could be detected.

The reduction of nitrate showed marked variation in the different cultures. Some showed only slight evidence of nitrite formation, others showed marked nitrite formation, while some of the cultures reduced the nitrate to ammonia.

The true thermophiles belonging in the group of spore-forming bacteria differed in their morphologic and biologic character to such a degree that satisfactory classification was not possible.

An attempt has been made to classify the different types of thermophilic bacteria and a comparison of the results obtained showed that amongst the more than sixty cultures studied, at least seven different rod forms and one thread form were

encountered that may be classed as true thermophiles, while one type of rod was encountered that may be regarded as a facultative thermophile.

The characters of the nine different types studied are given in tabular form on page 304.

The facultative thermophiles studied appeared to be more closely related to each other in that they formed short chains of rods with rounded ends having small oval central spores. These cultures all liquefied gelatin, fermented starch, and reduced nitrate to ammonia. Their action on milk showed at first a slight acidity followed by a slight alkalinity and slow peptonization of the casein. These cultures appeared to belong to a single species.

The true thermophiles resisted a temperature of 100°C., to an unusual degree varying from two to eight hours, so that it appears probable that the optimum temperature for growth is related to the heat resisting powers of the spores.

The facultative thermophiles with their lower optimum temperature for growth also showed a lower resistance to the boiling temperature. This group of bacteria was killed in from one-fourth to one hour at 100°C.

Two cultures of thread-form organisms were studied which had thermophilic properties. These two cultures represented two distinct varieties in their biological activities. On agar they showed a distinct white, mealy growth after several days and this fact has led to their classification with *Nocardia*. These two cultures differed in their action on milk and on starch solution, the one did not change milk but fermented starch to maltose, the other produced an acid reaction in milk with coagulation and subsequent digestion of the casein but only very slight action on starch. Both cultures liquefied gelatin but neither reduced nitrate. The maximum temperature of growth was about 70°C., and both were killed at 100°C., in two hours.

Although the thermophilic bacteria are widely distributed in nature it is evident that their activities can be brought into play under exceptional conditions. These organisms have been found in thermal springs where temperature conditions

	TRUE THERMOPHILES										FACULTATIVE
	Type 1	Type 2	Type 3	Type 4	Type 5		Type 6	Type 7	Type 8		Type 9
					Var. a	Var. b			Var. a	Var. b	
Source of organism	Dust and contaminated milk medium	Dust and soil	Dust, soil horse manure	Dust, soil, horse manure	Dust, g. pig feces, horse manure	Dust, cheese, g. pig feces	Horse manure	Horse manure	Rabbit's stomach	Contamination on agar	Dust, soil, horse manure
Form	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Thread	Thread	Rod
Motility	+	+	+	+	+	+	+	+	−	−	+
Size in microns	0.6-0.8 x 3-4	0.5-0.7 x 2-3	0.6-0.8 x 4-5	0.6-0.8 x 2-4	0.2-0.4 x 2-3	0.2-0.4 x 2-3	0.5-0.6 x 2-2.5	0.3-0.4 x1.5-2			0.5-0.7 x2-3
Grouping	Chains	Chains	Singly	Singly	Singly	Singly	Singly	Singly			Short chains
Location of spores	Central	Central	Central	Polar	Polar	Polar	−	−	Conidia	Conidia	Central
Spores larger or smaller diameter than rods	Smaller	Greater	Greater	Greater	Greater	Greater	−	−			Smaller
Gelatin liquefied	+	−	+	+	+	−	+	−	+	+	+
Nitrates reduced	NH_3	N_2O_3	±	N_2O_3	N_2O_3		±	N_2O_3	±	±	NH_3
Starch reduced	+	±	+	+	±	±	−	−	+	−	+
Action in litmus milk	Aid coagulation	Slight acidity	Slight acidity	Slight acidity	Slight acidity	Slight acidity	Slight acidity	Slight acidity	Slight acidity	Acid coagulation and digestion	Acid coagulation and digestion

Minimum temperature.....	50–60	37	37–50	50	37	37	37	37	60	60	20–37
Maximum temperature.....	75–80	70	70–75	75–80	60–70	60	60	60	70	70	50–60
Thermal-death point at 100° C. in minutes.	400	300	200	180	120	60	5	5	120	120	15–60

favor their development. In the surface layers of the soil in the temperate and torrid zones the temperature frequently rises to a point where they can develop and it is probable that they find an important field for action under these conditions.

There are other conditions in nature where it is probable that these organisms find suitable environment for their activities. In the fermentation of silage temperatures have been recorded as high as 55°C., but more commonly the temperature ranges between 35 and 45°C. The production of heat is probably not due to chemical changes in the fodder, by the tissue cells, but primarily to the activities of the thermophilic bacteria. Under these conditions it seems likely that the facultative thermophiles begin the fermentation processes and when the temperature rises to a sufficient degree then the true thermophiles begin to develop. Their ability to act on carbohydrates and protein indicates that they probably find sufficient food for development in various fodder crops.

The spoiling of hay and other fodder crops and of grain, when insufficiently cured, is no doubt due largely to the action of the thermophilic bacteria. The charring effect noticed in piles of improperly cured hay and fodder and in manure is probably caused by the thermophilic bacteria.

The conditions of temperature under which the thermophilic bacteria thrive are paradoxical when we note that the optimum temperature of growth is at about the temperature at which ordinary egg and serum albumin begin to coagulate. The maximum temperature of growth is decidedly above the temperature at which egg and serum albumin coagulate. This phenomenal characteristic may be due to the reaction of the medium in which the bacteria are growing or to the mineral content of their own protoplasm.

The investigation was interrupted in 1917 on entering the government service and the cultures were discarded. This accounts for the lack of details that would be desirable but it was thought advisable to publish the results that had been noted.

STUDIES IN THE METABOLISM OF ACTINOMYCETES

II

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Received for publication December 2, 1918

4. THE GROWTH AND ACTION OF ACTINOMYCETES UPON EGG-MEDIA

Egg-media were made the subject of special study on account of the very excellent growth of the organisms upon them and also on account of the fact that the liquefaction of the medium is an indication of the proteolytic action of the organisms. Three media were used, namely:

1. Whole egg. The eggs were sterilized on the surface, contents transferred into sterile containers, mixed with sterile spatula, then tubed, slanted, coagulated and sterilized in the Arnold at 75° to 90° for three consecutive days.

2. Lubenau's egg-medium, consisting of a mixture of 4 to 6 volumes of thoroughly mixed eggs with 1 volume of nutrient broth containing 5 per cent of glycerin. Coagulation and sterilization as before.

3. Petroff's (1915) medium containing 2 parts of well beaten eggs, one part of meat juice (15 per cent glycerin) and gentian violet in strength of 1: 10,000. The medium was tubed, coagulated and sterilized as before.

A number of Actinomycetes isolated from the soil, wounds and other sources, and grown upon a synthetic medium, were inoculated upon these 3 media, which were then incubated at 37°. An excellent growth was obtained with nearly all the organisms in twenty-four to forty-eight hours.

The organisms grew almost equally well on all the three media, producing a similar growth and similar reactions, so that the first medium appeared just as satisfactory as the other two.

But, for the isolation of the organisms from wounds, the Petroff's medium would probably prove best since the gentian-violet would eliminate many contaminations. Glycerin which, as will be shown later, forms a good source of carbohydrate for these organisms, seems to be unnecessary in the egg-medium since the egg alone probably supplies all the necessary constituents for the organisms. Gentian-violet, in 1:10,000 concentration, has no inhibitive effect upon at least most Actinomycetes.

Without taking up the cultural description of the organisms upon the media, we might point out some characteristic chemical changes produced by the organisms. *A. madurae*, *A. griseus*, 128, *A. hominis* produced a liquefaction of the coagulated medium, indicating that these organisms are able to hydrolize the coagulated egg-albumin. It will be noticed that the organisms that are able to do this are the ones found to be most active proteolytically upon milk proteins and coagulated blood serum and which hemolized the blood in blood agar.

A second fact noted was the production of a deep brown to violet and almost black pigment by a number of species, such as *A. poolensis*, *A. bobili*, *A. viridochromogenus*, *A. reticuli*, 120, 205, *A. aureus*, *A. scabies*, 215 and 218. It will be observed that the organisms producing this brown to black pigment upon egg-media are the same ones that produced a brown pigment when grown upon milk. Pergolo (1917) stated recently that the ability of chromogenic bacteria to produce pigments when grown on Lubenau's medium is due to the presence of the yolk of egg; the ether-soluble part of the yolk favors the most chromogenesis. This is no doubt true. It is probable that these organisms produced the enzyme tyrosinase which acting upon the egg constituents (previously split) will result in the production of the pigment.

Summary

1. Egg-media allow a good growth of Actinomycetes to take place in a short period of time.

2. The addition of gentian-violet in a concentration of 1:10,000 to the medium has no detrimental effect upon these organisms.

3. The liquefaction of the coagulated egg is characteristic of the strongly proteolytic organisms.

4. The production of a brown pigment is characteristic of certain organisms.

5. THE ACTION OF ACTINOMYCETES UPON GELATIN

A solution of gelatin in water, with or without other nutrient constituents, is extensively used by bacteriologists to demonstrate the fact whether a certain organism is proteolytic in nature or not. The liquefaction of the gelatin is due to a proteolytic enzyme produced by the organism. Some investigators (Pollak) claimed that this enzyme, *gelatinase*, is distinctly different from other proteolytic enzymes since its action results only in the liquefaction of the gelatin, while its action upon the hydrolysis of proteins is very slight. Ascoli and Neppi (1908) have shown that the favorable action of the enzyme isolated by the method of Pollak (1905) upon the gelatin and not upon other proteins is not specific for the enzyme, but is due to the special conditions of the medium. The liquefaction of the gelatin has been usually taken as a sufficient index of the proteolytic action of the microorganisms or enzymes. Some investigators have gone further however and determined also the actual digestion of the gelatin by measuring the nitrogen precipitated by tannin, while others have obtained the amount of amino acid nitrogen produced, using the "formaldehyde" method of Sörensen.

Some of the Actinomycetes are found to be very active proteolytically. Attention has already been called to this fact in previous papers. Nearly all the Actinomycetes, with very few exceptions (*A. asteroides*), are found to liquefy gelatin at an earlier or later period of their growth, so that the liquefaction of gelatin as such could not be used for the differentiation of the species. Since they differ only in the rapidity of liquefaction, confusion would often arise, due to the fact that some forms were reported to be unable to liquefy the gelatin, while, had a longer period of incubation been allowed, the liquefaction would have been apparent.

Beijerinck (1900) demonstrated the fact that the *A. chromogenus* species produce *quinone* which acts upon the gelatin and converts it into an insoluble form. The production of a brown pigment upon the gelatin is due to the formation of quinone by the organisms in question.

Krainsky (1914) has recently shown that most Actinomycetes grow on gelatin media producing liquefaction in most cases; the chromogenus-species producing an insoluble gelatin compound. Waksman and Curtis (1916) have shown that all the Actinomycetes that they isolated from the soil liquefied gelatin but that the rapidity of liquefaction was distinctly different for different organisms. The production of a brown pigment was characteristic of some species.

The nature of the growth of the Actinomycetes upon gelatin, the chemical changes produced in the gelatin as a result of the growth of the organisms and the constancy of these characters for a given species will be reported on in this paper.

The cultures were the same studied in the previous investigations. The organisms were grown upon Czapek's solution agar, then inoculated upon the gelatin. One hundred and fifty grams of "Gold Label" gelatin were dissolved in a liter of distilled water, then tubed and sterilized in flowing steam on three consecutive days. The adjustment of the reaction did not seem to have an important effect upon the growth and liquefaction; but for the purpose of having conditions more constant, the pH of the gelatin solution was adjusted to 7.0 before sterilization, using the colorimetric method of Clark and Lubs (1917). At first the organisms were grown in colonies on plates and the diameter of the liquefied zone measured, but it was found that the growth in a standard size tube and subsequent measurement of the depth of liquefaction gave more comparable results. The latter method could not bring out the typical growth of the organism, but gave a good means for comparing the amount of liquefaction.

The results reported in table 1 include the information obtained three years ago with the same organisms, soon after their isolation from the soil, also the results of the action of the organisms upon gelatin after they had been grown for three years on artificial, mostly synthetic, culture media.

The incubation temperatures were 20° and 25°.

The amino nitrogen was determined at the end of forty days incubation, using the micro-method of Van Slyke (1914). In a case where the gelatin was not entirely liquefied, the liquid portion was used for the analysis.

A study of table 1 will reveal the fact that the liquefaction of the gelatin is a rather constant property of the organisms and can be used in their differentiation. But different methods and different temperatures of incubation may interfere with thoroughly concordant results. This is due to the fact that the different species will grow with a different degree of rapidity on different media and at different temperatures. The rapidity of liquefaction of gelatin may therefore be used only in supplying some additional information as to the identification of the organism, but may not be considered of absolute value. The production of a brown pigment, chiefly in the unliquefied portion of the gelatin, brought out more clearly by the use of the plate method, is more characteristic of the organism, but even here certain few organisms (*A. albus*) are found which originally produced a brown pigment but later lost that property, after they were grown for three years on artificial culture media. The production of aerial mycelial is also not absolutely constant. A few organisms may produce aerial mycelium at one time and not at another. These discrepencies can be readily explained by the variability of the same organism upon the same culture medium. If a large enough number of cultures of the same organism were studied, to eliminate the individual variation of the same strain on the same medium, these characteristics might become more definite although even then we would expect certain changes in the organism on transfer and continuous growth upon artificial culture media. But when we are studying only one or two tubes at a time, the possible error is very great. The organism cannot therefore be studied and identified on one or two media, most of which, as commonly used by bacteriologists and plant pathologists, are not standard in composition, such as nutrient agar, cornmeal agar, etc. A large amount of information has to be obtained for each individual organism, grown on standard media and studied in detail as to its biochemical characteristics,

TABLE 1

The growth of actinomycetes upon gelatin

NAME OF ORGANISMS	FALL 1915, 20°			FALL 1918, 20°				FALL 1918, 25°		
	Liquefaction	Pigment	Aerial mycelium	Liquefaction	Pigment	Aerial mycelium	NH_3N, mgm. in 10 cc.	Liquefaction	Pigment	Aerial mycelium
				mm. in 30 days			*Control = 7.25*	*mm. in 4 days*		
A. violaceus-ruber	Slow	None	White	20	None	None	36.0	8	Bluish	None
A. violaceus-caesari	Rapid	None	None	8	None	None	21.0	10	None	None
A. californicus	Slow	None	White-gray	6	None	Gray	18.2			
A. scabies	Rapid	Brown	None	10	Brown	None	42.0	12	Brown	None
A. exfoliatus	Slow	None	Gray-brown	10	None	Brown	46.2	12	None	Gray
A. diastaticus	Rapid	None	White	40	None	White	50.0	20	None	White
A. albus	Rapid	Brown	White	5	None	White		4	None	White
A. reticuli	Rapid	Brown	None	12	Brown	White		2	Brown	White
A. 120	Rapid	Brown	Pink	36	Brown	Pinkish		30	Brown	Pink
A. citreus	Rapid	None	White	9	None	None	31.5	2	None	None
A. griseus	Rapid	None	White	50	None	White	54.6	25	None	White
A. alboflavus	Rapid	None	None	5	None	None		15	None	None
A. verne	Rapid	None	None	12	None	None	14.4	10	None	None
A. albosporeus	Rapid	None	Reddish	34	None	Reddish		12	None	Reddish
A. bobili	Rapid	Brown (late)	None	35	Brown	None	51.0	8	Brown	None
A. lipmanii	Rapid	None	White-gray	10	None	None	32.5	10	None	None
A. rutgersensis	Rapid	None	None	30	None	None	33.5	5	None	White
A. aureus	Rapid	Brown	White	4	Brown	None	14.8	5	Brown	None

A. fradii	Rapid	None	White	4	None	White (scarce)	16.8	8	None	None
A. lavendulae	Slow	Brown	None	None	Brown	None		None	Brown	None
A. madurae				36	None	None	43.2	10	None	White
A. hominis				16	None	None	51.0	10	None	None
A. bovis				30	None	None	19.2	10	None	None
A. asteroides				None	None	None		None	None	None
96				13	Golden	White		20	Golden	White
128	Rapid	None	White	18	None	Ash-gray	45.5	20	None	Gray
161	Slow	None	White	Start	None	White		None	None	White
202				6	Brown	None		3	Brown	None
101	Medium	Brown	Green	10	Brown	Green	40.0	2	Brown	Green
205				15	Brown	White	30.0	10	Brown	White
206				40	None	None		16	None	None
215				6	Brown	Scant		20	Brown	None
214				20	None	None		25	None	None
A. poolensis				8	Golden	None	33.0	10	None	None
168	Medium	Brown	White	15	Red	White	35.5	8	Brown	White

and even then we will have to study species not by themselves but in their group relationship.

The amino nitrogen content of the liquefied gelatin points distinctly to the proteolytic properties of the organisms. Although at 25° nearly all the cultures, with the exception of *A. lavendulae*, *A. asteroides*, *A. aureus*, 96, and one of two others, not reported in the Table, were fully liquefied in twelve to twenty-five days, the amino nitrogen content of the liquefied gelatin was distinctly different. This indicates that, although some organisms may liquefy the gelatin, the hydrolysis is not carried very far. For example, the gelatin of *A. violaceus-caesari*, *A. verne*, *A. bovis*, *A. aureus*, and *A. fradii* cultures shows a comparatively small increase in the amino nitrogen content of the gelatin, while the cultures of *A. griseus*, *A. diastaticus*, *A. exfoliatus*, *A. bobili*, *A. madurae*, *A. hominis*, 128 contain large quantities of amino nitrogen. These results, when compared with those reported in the previous papers, indicate that the organisms most active proteolytically on milk and blood serum are also most active upon gelatin, while those least active upon those natural protein-rich media are among the least active on gelatin.

These results would seem to point to the fact that the liquefaction of gelatin may not necessarily indicate a strong proteolytic action, although the proteolytically active organisms liquefy gelatin very rapidly, due to the hydrolysis of this compound. Before we know more definitely the physical process of gelatin liquefaction and the accompanying chemical changes, if there are any (as in the case of warming the gelatin), we cannot expect to correlate the hydrolysis and liquefaction of gelatin in more than a probable way. Berman and Rettger (1918) have recently stated that the ability of an organism to liquefy gelatin is not in itself a proteolytic or even a gelatinolytic function, for some organisms which can liquefy gelatin are unable to carry the change beyond the gelatose stage, and fail to decompose "purified" proteose and casein.

The production of enzymes liquefying and hydrolyzing gelatin, goes on even when the organisms are grown upon synthetic media. Several Actinomycetes were grown on Czapek's

synthetic solution, then filtered. Both filtrate and mycelium, treated by the "acetonedauerhefe" method, were able to liquefy the gelatin in twenty-four to ninety-six hours with an increase in the amino nitrogen content.

The organisms producing the brown pigment upon gelatin usually produce a similar pigment when grown upon other complex nitrogenous media, such as egg, blood and blood serum medium. This is probably due to the production of an oxidizing substance such as quinone demonstrated by previously named investigators upon gelatin. The action of these organisms upon gelatin results first in a slow liquefaction of the gelatin, with a small increase in the amino nitrogen content, indicating hydrolysis. Later, particularly with the production of an intense brown pigment and not at a very high temperature (about 20° or below), the liquefied gelatin solidifies again, as in the case of a few chromogenus species, including *A. lavendulae* and 205.

6. THE UTILIZATION AND TRANSFORMATION OF DIFFERENT CARBON COMPOUNDS BY ACTINOMYCETES

Several important references are found in the literature on the utilization of carbon compounds by Actinomycetes. Beijerinck (1900) stated that these organisms produced diastase; the production of this enzyme was also demonstrated by Sames (1900), starch being reduced only to the point of giving the erythro-reaction and no reducing sugars being produced. Caminiti (1913) found that the Actinomycetes produced invertase and diastase. Münter (1913) made a rather thorough study of the utilization of carbon compounds using ammonium nitrate as a source of nitrogen. Glycerin, levulose, glucose, galactose, mannite, starch and lactose proved to be good sources of carbon for all Actinomycetes; only one organism made a poor growth on lactose; arabinose was well utilized by some and not by others; sucrose was used only by certain species (chromogenus types and one other); only one organism could assimilate inulin. Oxalic, tartaric and hippuric acids in the form of sodium or calcium salts, were utilized to only a very small extent or not at

all, while succinic and citric acids offered good sources of carbon. With the introduction of the oxy groups into the molecule of succinic acids, its utilization was found to decrease since the growth diminished with malic acid and almost ceased entirely with tartaric acid. Acetic acid was utilized to some extent, while lactic acid formed a good source of carbon.

Krainsky (1914) stated that Actinomycetes made a good growth upon media containing mono-, di- and poly-saccharides and salts of organic acids. $CaCO_3$ was produced from glucose and calcium malate. Glucose was oxidized with the production of CO_2, leaving the medium acid. A few species were found by Krainsky to produce invertase and most of them produced a rather active diastase, while maltose and raffinose were not split. Cellulose was used by the Actinomycetes with different rapidity.

Salzmann (1902) obtained a good growth of *Actinomyces odorifer* upon many carbohydrates, particularly glycerin, in neutral, acid or alkaline solutions. The calcium salts of organic acids containing one carboxyl group proved to be poor sources of carbon, while good growth took place, when a second carboxyl group was present.

Fousek (1912) stated that glucose, lactose, starch and cellulose formed good sources of carbon for Actinomycetes, while urea and uric acid could not be used as sources of carbon, although they were good sources of nitrogen.

The fermentation of carbohydrates is commonly used by bacteriologists as a method of differentiating different bacterial species and even groups. The information thus obtained together with a mere microscopic and macroscopic study is often sufficient for a proper identification. The change in reaction (usually acid production) and production of a gas are taken as indications of the fermentation, the two reactions not necessarily going hand in hand.

To find out whether this method could also be applied to the study of Actinomycetes, the ordinary medium and bacteriological methods were used at first. Sterile beef infusion (500 grams beef per liter of water, boiled, filtered and sterilized) was inoculated with *B. coli-communior* and incubated at 37° for twenty

hours. The material was then boiled for ten minutes, adjusted to pH = 7.6 to 7.7, boiled again, filtered and sterilized. One per cent of the proper carbohydrate and 1 per cent of Andradé (Holman, 1914), indicator were added to the sterile sugar-free broth, and the medium was then distributed in fermentation tubes. The tubes were steamed in the Arnold for thirty minutes, incubated to insure sterility and inoculated with the Actinomycetes. This method of precedure, so much used in bacteriological work, proved to be unsatisfactory for the study of the Actinomycetes. These organisms do not form any gas from the different carbohydrates, therefore the fermentation tube is unnecessary; they do not produce any acid, as will be shown elsewhere, but have a tendency to produce a decrease of the hydrogen-ion concentration of the medium, therefore the Andradé indicator, which registers only acid production is of no value in this case. Furthermore, since the meat infusion itself will allow a growth of the Actinomycetes, the effect of the carbon compounds cannot be differentiated properly.

An inorganic medium standard in composition had therefore to be selected for this study. Since the Czapek's solution agar had been extensively used for the study of the Actinomycetes by the writer and found to give satisfactory results, this solution was also used as a basis for the following experiments. One gram K_2HPO_4, 0.5 gram $MgSO_4$, 0.5 gram KCl, 0.01 gram $FeSO_4$ and 2 grams $NaNO_3$ were added to every liter of distilled water. This was used as a basis to which 2 per cent of the following carbon compounds were added: arabinose, glucose, lactose, sucrose, maltose, mannite, glycerin, starch and the sodium salts of acetic, malic, tartaric, oxalic and lactic acids. All of the materials were chemically pure, either Kahlbaum's or Merck's. Cellulose was added in two forms: a small piece of Whatman filter paper was introduced into each tube, the solution containing no other source of carbon, or 1 cc. of 0.5 per cent cellulose suspension prepared by the method used by McBeth (1916) and others. The media were tubed, sterilized at 15 pounds pressure for fifteen minutes, incubated to insure sterility and inoculated with several cultures of Actinomycetes, grown on the

Czapek's agar. The cultures were incubated for fifteen days. At the end of that period the cultures were studied as to the growth characters and the effect of different carbon compounds upon the change of reaction, as measured by the hydrogen-ion concentration, using the phenol-sulphonphthalein series of indicators of Clark and Lubs (1917), and upon the production of nitrites; these were determined by the Griess colorimetric method (1879).

The results obtained from the study of 27 organisms using 14 different carbon compounds were tabulated. The media to which arabinose, glucose, maltose and lactose were added were distinctly acid due to the effect of the high pressure in sterilization upon these sugars.

The results presented in table 2 yield themselves readily to comparisons. It might be argued that since the hydrogen-ion concentration of the arabinose, glucose, maltose and lactose media was higher than that of the others, being distinctly acid, due to the effect of sterilization upon these sugars, the comparative values may be affected. But this factor is not of very great importance, as will be pointed out in one of the following papers of this series, if the reaction does not extend beyond certain limits of acidity and alkalinity. It was also recently demonstrated by Gillespie (1918) that, although on media with an hydrogen-ion concentration of pH = 5.2 and less the growth of *Actinomyces chromogenus* (*A. scabies*) was slower and generally less vigorous than in the less acid media, the difference between the media having an hydrogen-ion concentration of pH = 5.8, 6.1 and 7.2 was hardly noticeable. The hydrogen-ion concentration of the arabinose, glucose and lactose media was about pH = 6.2–6.3, so that the effect of the reaction would probably be of no great importance in this case.

Upon adding the figures designating the amount of growth for all the organisms studied and dividing the sum by the number of organisms, we obtain certain factors, which designate the utilization of the different carbon compounds by the Actinomycetes as a group. The factors are for arabinose 1.13, glucose 3.0, lactose 2.69, sucrose 1.93, maltose 2.67, mannite 2.17, glycerin 2.37,

TABLE 2

The utilization of different carbon compounds by actinomycetes and the effect of these upon the hydrogen ion concentration of the medium and reduction of nitrates

NAME OF ORGANISMS	ARABINOSE					GLUCOSE					LACTOSE					SUCROSE					MANNITE				
	Gr*	AM	Col†	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit
Control	0	0	0	6.2	0	0	0	0	6.3	0	0	0	0	6.2	0	0	0	0	7.0	0	0	0	0	7.2	0
A. violaceus-ruber	1	0	1	6.3	5	5	2	1	7.0	5	5	2	1	7.1	5	0	0	0	7.0	3	3	2	1	7.2	5
A. violaceus-caesari	1	0	1	6.3	1	3	0	1	6.4	0	1	0	2	6.3	0	2	0	2	6.9	1	1	0	2	7.2	1
A. albus	2	1	1	6.4	0	3	2	1	6.4	0	3	2	1	6.9	1	2	0	1	6.9	1	3	2	1	7.1	1
A. aureus	0	0	0	6.2	1	4	1	1	6.5	1	3	2	1	6.4	1	2	0	2	7.4	0	2	1	1	7.2	1
A. exfoliatus	4	2	1	6.8	0	4	2	1	7.0	0	4	2	1	6.8	1	5	3	1	7.1	1	3	1	1	7.0	1
A. griseus	3	2	2	6.5	0	4	2	2	6.9	2	3	2	2	7.1	0	1	2	2	7.4	0	3	2	2	7.7	3
A. scabies	4	2	1	6.7	1	2	0	1	6.4	0	4	1	1	6.9	1	2	0	1	6.9	0	3	1	1	7.6	1
A. verne	0	0	0	6.2	0	1	0	2	6.3	1	2	0	1	6.4	3	2	0	2	6.8	3	3	0	1	7.2	4
A. lipmanii	0	0	0	6.2	1	3	0	2	7.0	0	3	3	2	7.0	2	3	0	3	6.8	0	3	2	2	7.4	2
A. virido-chromogenus	3	2	2	6.7	2	4	3	2	7.1	2	3	3	2	6.9	3	2	3	2		2	3	2	2	7.3	2
A. bobili	0	0	0	6.2	0	2	0	2	6.5	0	3	0	1	6.7	1	2	0	2	6.9	1	0	0	0	7.1	0
A. poolensis	0	0	0	6.2	0	1	0	2	6.4	0	2	0	2	6.3	0	1	0	2	7.0	1	1	0	2	7.2	1
A. diastaticus	4	2	3	6.9	1	4	2	2	7.1	0	4	4	2	6.8	1	2	2	1	7.4	0	4	2	2	7.4	1
A. fradii	3	1	3	6.5	2	4	2	2	6.5	0	3	4	1	6.8	1	1	0	2	7.3	1	3	1	1	7.3	1
A. roseus	1	2	0	6.4	4	3	0	1	7.1	5	1	0	2	6.7	1	2	1	1	7.3	3	1	0	1	7.0	1
A. albosporeus	0	0	0	6.2	0	3	2	1	6.5	1	2	2	1	6.5	1	2	0	1	7.0	0	4	0	1	7.4	3
A. asteroides	0	0	0	6.2	0	4	0	1	6.7	3	2	2	1	7.0	2	2	1	2	6.8	2	0	0	0	6.4	1
A. madurae	0	0	0	0.0	0	0	0	0	0.0	0	0	0	0	0.0	0	1	0	2	7.6	1	0	0	0	0.0	0
A. hominis	0	0	0	0.0	0	0	0	0	0.0	0	3	2	1	7.5	3	2	0	2	7.3	0	0	0	0	0.0	0
A. bovis	0	0	0	0.0	0	0	0	0	0.0	0	2	2	0	6.8	5	1	0	1	7.2	5	0	0	0	0.0	0
104	0	0	0	6.2	0	1	0	2	6.4	0	1	0	2	6.3	0	1	0	2	7.6	0	1	0	2	7.0	0
120	0	0	0	6.2	0	4	3	2	6.9	4	2	0	1	6.3	1	1	0	2	7.6	1	0	0	0	7.0	4
128	0	0	0	6.2	0	2	2	1	6.4	0	2	0	1	6.4	0	1	1	1	6.9	0	1	1	1	7.1	0
154	0	0	0	6.2	0	3	0	1	6.4	0	4	0	1	6.4	0	2	0	2	7.0	1	1	0	2	7.3	0
168	0	0	0	6.2	0	4	2	1	7.2	2	4	2	1	6.9	1	2	1	1	7.0	1	5	2	1	7.5	2
205	0	0	0	6.2	0	1	0	2	6.3	0	2	0	2	6.3	0	2	0	2	6.8	0	1	0	2	7.1	0
215	1	0	2	6.4	4	3	2	1	6.4	1	3	2	1	6.9	3	3	1	1	7.2	1	3	1	1	7.2	1

*Gr = growth; AM = aerial mycelium; Col = colonies; pH = index of hydrogen ion concentration; Nit = nitrites; 0 = absence; 1 = faint or trace; 2 = fair; 3 = good; 4 = very good; 5 = excellent.

† 1 Type = round colonies; 2 type = floccules; 3 type = mass of growth; 2 for the organic acids indicates a small amount of growth on the bottom of tube.

TABLE 2—*Continued*

NAME OF ORGANISMS	GLYCERIN					STARCH					CELLULOSE					CELLULOSE SUSPENSION					MALTOSE				
	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit
Control	0	0	0	7.1	0	0	0	0	7.0	0	0	0	0	7.1	0	0	0	0	7.1	0	0	0	0	6.2	0
A. violaceus-ruber	3	1	1	6.7	5	5	2	1	7.0	5	2	0	1	7.1	2	3	1	1	7.2	3	4	2	1	7.2	5
A. violaceus-caesari	0	0	0	7.4	0	3	0	1	7.0	1	2	0	1	7.1	0	3	0	2	7.5	1	2	0	2	5.6	0
A. albus	3	1	1	7.1	0	3	1	1	7.2	1	0	0	0	7.3	0	0	0	0	7.1	0	3	0	2	6.0	0
A. aureus	3	1	1	7.0	1	2	1	1	7.1	1	0	0	0	7.0	0	0	0	0	7.3	0	3	0	2	6.2	3
A. exfoliatus	3	1	1	7.0	0	4	2	1	7.3	1	0	0	0	7.0	0	1	0	2	7.2	0	4	3	3	7.6	1
A. griseus	2	1	2	7.2	1	4	2	2	7.4	2	1	0	2	7.6	0	0	0	0	7.6	1	2	0	2	6.0	0
A. scabies	3	0	1	7.4	2	3	1	1	7.4	1	1	0	1	7.4	0						2	0	2	6.2	2
A. verne	1	0	2	7.0	1	1	0	2	6.9	1	3	0	1	7.2	1	1	0	2	7.3	1					
A. lipmanii	3	2	2	7.5	3	3	2	2	7.3	4	0	0	0	7.4	1										
A. virido-chromogenus	2	2	2	7.3	3	3	2	2	7.5	2	1	1	1	7.1	1	3	0	1	7.2	1	4	0	1	5.6	2
A. bobili	3	0	1	7.4	1	3	1	2	7.1	1	3	0	1	7.4	0	0	0	0	7.3	0	2	0	1	6.2	0
A. poolensis	4	0	1	7.2	1	3	0	1	7.3	3	2	0	1	7.1	0	0	0	0	7.3	0	1	0	2	6.4	0
A. diastaticus	4	4	2	7.4	1	4	1	2	7.5	1	0	0	0	7.1	0	0	0	0	7.3	0	3	4	1	8.0	0
A. fradii	3	1	1	7.2	1	4	1	1	7.1	2	0	0	0	7.5	0	0	0	0	7.2	0	2	0	2	6.2	0
A. roseus	2				4	3	0	1	7.0	5	1	0	1	7.7	4										
A. albosporeus	1	0	2	7.1	0	4	1	1	7.5	3	0	0	0	7.2	0	0	0	0	7.3	1	1	0	2	6.6	0
A. asteroides	2	1	1	6.7	4	0	0	0	6.9	0	1	0	1	6.7	1	1	0	2	7.1	1	3	0	2	6.2	4
A. madurae	1	0	2	7.2	1	0	0	0								0	0	0	7.1	1	1	0	2	6.2	1
A. hominis	5	3	1	7.6	3	0	0	0								1	0	2	7.5	0	4	3	1	7.4	4
A. bovis	1	2		7.2	5	0	0	0								0	0	0	7.2	0	1	0	2	6.5	2
104	1	0	2	7.0	0	1	0	2	6.8	0	1	0	1	7.1	0	1	0	2	7.2	0	2	0	2	6.0	0
120	3	1	3	7.3	1	5	2	2	7.0	5	3	0	1	7.1	1	1	0	2	7.2	1	4	2	1	7.1	4
128	1	1	1	7.1	0	3	1	1	6.8	0	1	0	1	7.0	0	0	0	0	7.2	0	3	4	1	6.2	3
154	2	0	2	7.2	1	2	0	1	7.1	1	2	0	1	7.2	0	0	0	0	7.1	0	2	0	2	6.2	0
168	4	1	1	7.5	4	3	1	1	7.2	1	2	0	1	7.1	0	1	0	2	7.1	0	3	2	1	6.6	3
205	1	0	2	7.0	1	4	1	1	7.3	1	2	0	2	7.1	1	0	0	0	7.3	0	4	3	1	7.2	1
215	3	1	1	7.3	1	4	2	2	7.6	1	2	0	1	7.1	1	2	1	1	7.1	1	4	1	1	7.0	3

TABLE 2—*Concluded*

NAME OF ORGANISMS	SODIUM ACETATE					SODIUM LACTATE					SODIUM TARTRATE					SODIUM OXALATE					SODIUM MALATE				
	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit	Gr	AM	Col	pH	Nit
Control	0	0	0	7.3	0	0	0	0	6.6	0	0	0	0	7.3	0	0	0	0	7.8	0	0	0	0	7.8	0
A. violaceus-ruber	1	0	2	7.8	5						1	0	2	7.7	1	0	0	0	7.7	2	1	0	2	8.1	3
A. violaceus-caesari	1	0	2	7.8	0	1	0	2	6.6	1	1	0	2	7.8	1	1		2	7.6	0	1	0	2	8.0	1
A. albus	1	0	2	7.7	0	2	0	2	7.0	1	0	0	0	7.8	1	1	2		7.6	0	0	0	0	8.0	1
A. aureus	1	0	2	7.0	0	1	0	2	6.2	0	1	0	2	7.7	0	0	0	0	7.6	0	1	0	2	7.8	0
A. exfoliatus	1	0	2	7.1	0	1	0	2	6.4	0	2	0	2	7.6	0	1	0	2	7.6	0	1	0	2	7.9	0
A. griseus	1	0	2	8.2	0						1	0	2	7.8	1	1	0	2	7.4	1	3	2	3	8.8	3
A. scabies	1	0	2	7.0	0	1	0	2	6.6	0	1	0	2	7.4	0	1	0	2	7.5	0	1	0	2	7.6	0
A. verne						1	0	2	7.0	1	0	0	0	8.0	1	1	0	2	7.7	1	0	0	0	8.0	1
A. lipmanii																									
A. virido-chromogenus	1	0	2	7.5	2	2	0	1	6.8	0	2	0	1	7.8	1	1	0	2	7.5	1	2	0	1	7.7	1
A. bobili	2	0	2	7.4	0	1	0	2	6.6	1	0	0	0	7.8	0	0	0	0	7.6	0	1	0	2	8.0	0
A. poolensis	0	0	0	7.3	0	0	0	2	6.6	0	0	0	0	7.5	0	1	0	2	7.7	0	1	0	2	8.1	0
A. diastaticus	0	0	0	7.6	0	1	0	2	6.6	0	0	0	0	7.5	0	1	0	2	7.6	0	1	0	2	8.1	1
A. fradii	1	0	2	7.3	0	1	0	2	6.6	0	1	0	2	7.8	0	0	0	0	7.5	0	1	0	2	8.0	0
A. roseus																									
A. albosporeus	1	0	2	7.3	1	1	0	2	6.5	1	1	0	2	7.7	1	1	0	2	7.5	1	1	0	2	7.8	1
A. asteroides	1	0	2	7.6	2	2	0	2	7.7	4	2	2	1	7.5	2						1	0	2	7.7	2
A. madurae	1	0	2	7.4	1	2	1	1	6.6	1	1	0	2	7.7	1	1	0	2	7.5	1	1	0	2	8.0	1
A. hominis	1	0	2	7.5	0						1	0	2	7.8	1	1	0	2	7.8	1	1	0	2	8.6	1
A. bovis	1	0	2	7.4	2						0	0	0	7.6	0	1	0	2	7.7	1	1	0	2	8.0	0
104	1	0	2	7.7	0						1	0	2	7.7	0	0	0	2	7.5	0	1	0	2	8.2	0
120	2	0	1	8.0	1	1	0	2	6.6	1	1	0	1	7.7	1	1	0	2	7.6	2	1	0	2	8.3	1
128	1	0	2	7.3	1	1	0	1	6.6	0	1	0	2	7.7	0	1	0	2	7.7	0	0	0	0	8.0	0
154	0	0	0	7.4	0	1	0	2	6.6	0	1	0	2	7.8	0	1	0	2	7.6	0	1	0	2	8.0	0
168	1	0	2	7.4	0	2	0	2	6.6	0	2	0	2	7.7	0	1	0	2	7.4	0	1	0	2	7.7	0
205	1	0	2	7.9	1	0	0	0	6.6	0	0	0	0	7.8	0	1	0	2	7.6	0	1	0	1	8.6	3
215	2	0	1	8.0	2						1	0	2	7.8	1	1	0	2	7.6	1	1	0	2	7.8	1

starch 3.08, cellulose paper 1.25, cellulose suspension 0.75, sodium acetate 1, sodium lactate 1.16, sodium tartrate 0.88, sodium oxalate 0.79, sodium malate 1.00. Although these factors are not absolute in value, since they are affected not only by the presence or absence in the investigation of different individual organisms, but also by the periods of incubation and individuality of the recorder as well as by the inexact and abstract value of the estimate of growth. But the facts that these organisms were selected at random, that many representatives of the group were taken, a similar treatment used, and record of the amount of growth carefully made by one person at one time, will tend to give a certain value to these indications.

Starch was found to give the highest figure and it probably is one of the best sources of energy for most Actinomycetes. Glucose and lactose follow next, while maltose and glycerin occupy only the fourth and fifth places. That glycerin was not found to be such a good source of carbon for the Actinomycetes, as starch, glucose and lactose, may be due also to the source of nitrogen which affects the utilization of the carbohydrates, as was pointed out by Krainsky (1914), a point which will be taken up later.

Sucrose, cellulose, both paper and suspension, arabinose and the sodium salts of organic acids are rather poor sources of energy for the group as a whole as indicated by the factors of growth with $NaNO_3$ as a source of nitrogen. But, on comparing the action of individual organisms upon these carbon compounds, we find important differences between the sodium salts of the organic acids on the one hand and sucrose, arabinose and cellulose on the other. The sodium salts of the organic acids, particularly some of them, such as the malate, lactate and acetate can be used to some extent; the growth of the organisms upon these carbon compounds is very restricted indicating the fact that they do not offer very favorable carbon sources for the Actinomycetes. The growth of most organisms upon the liquid media containing these organic acids was limited to minute colonies or flakes in the bottom of the tubes, and only in few instances was a fair amount of growth obtained.

The amount of growth of an organism, with sucrose as a source of energy, depends entirely upon the ability of that organism to produce invertase, although even the organisms not producing invertase seem to use it to some extent. Cellulose and arabinose are used by only a few organisms, and this leads to the reduction of the growth factor for the whole group, although, when an organism does use these compounds, the growth may be quite abundant.

The reduction of nitrates to nitrites is a rather common property of the Actinomycetes, but the extent of reduction and even the very presence of it seems to depend on the nature of the carbon source present in the medium. When the figures representing the different quantities of nitrites produced (and these are again only relative) by the different organisms are added and the sum divided by the number of organisms, we obtain a factor for nitrite production by Actinomycetes for each source of carbon. These are for arabinose 0.92, glucose 1.13, lactose 1.42, sucrose 1.07, maltose 1.5, mannite 1.5, glycerin 1.66, starch 1.79, cellulose paper 0.54, cellulose suspension 0.46, acetate 0.83, lactate 0.53, tartrate 0.52, oxalate 0.5 and malate 0.84. Although the same criticisms can be applied to this method as to the measurement of the utilization of carbon compounds, the results obtained point to the fact that the method is not so inaccurate, when a large enough number of organisms are studied. A rather fair parallelism is obtained between the availability of the carbon compound as indicated by the growth of the organisms and the reduction of nitrates, pointing to the fact that the reduction is most active with the best growth, in the presence of a readily available carbon compound. Starch is again leading and is followed by glycerin, maltose, mannite, lactose and glucose. The interesting thing is the interchange of places between glycerin and glucose; while the growth factor of glucose was second and of glycerin fifth, the nitrite factor of glycerin was second and of glucose sixth. Arabinose, cellulose and the salts of the organic acids came last. The salts of the organic acids which gave higher growth factors gave also (except lactate) higher nitrite factors. This indicates that the process of nitrate

reduction is not a part of the energy utilization process, but accompanies the energy transformation of the organism. This question will be taken up in detail in a paper on nitrogen transformation by Actinomycetes.

As to the question of change in reaction, attention may be merely called here to the fact that the Actinomycetes, with very few exceptions, seem to be active in producing alkalinity in the medium, when $NaNO_3$ is the only source of nitrogen. An increase in acidity is observed only in few instances, while an increase in alkalinity is very apparent for most organisms. When we calculate the average for the hydrogen-ion concentration of the different organisms for one carbohydrate, we find that the organisms were most active in this respect in the starch medium. This seems to correspond with the occurrence of the best growth and largest quantities of nitrites. The organisms growing most rapidly, in the presence of a readily available carbon compound, reduce the nitrates very actively.

The source of carbon affects the pigment of the colony; thus, for example, the colonies of *A. fradii* are yellow in starch and arabinose, orange colored in glucose and white in glycerin. Having discussed the utilization of carbon compounds as a whole, we will take up now the specific action of Actinomycetes upon the three carbohydrates, sucrose, starch and cellulose.

Sucrose is used to some extent by nearly all the Actinomycetes studied and it forms the only source of energy in the Czapek's solution agar, used extensively by the writer for the study of this group of organisms. Only those organisms that produce invertase can make on this medium more than a rather poor growth. This fact is of great importance in the differentiation of the Actinomycetes, since media containing readily available constituents, such as nutrient agar, etc., allow these organisms to develop rapidly and very little differentiation can be made between the different organisms. A characteristic aerial mycelium is usually produced on the Czapek's solution agar, which is also very important in the identification of an organism. When glucose, starch or glycerin are substituted in place of sucrose in this medium, the growth of most organisms is much

quicker and more abundant, but in most cases not as characteristic. Only a few of the organisms were found to be able to invert the sucrose of the medium: these are *A. violaceus-caesari*, *A. verne*, *A. scabies*, *A. bobili*, 215 and 104. All these organisms, with the exception of the last make a very abundant growth upon the Czapek's solution agar, while the growth of the other organisms is restricted and aerial mycelium is produced early and readily. We can thus see that the amount of growth of an organism and the corresponding production of aerial mycelium may not be anything else but a result of the nutrition of the organism.

Two methods were used for the study of diastase production, the tube and plate method. In the first case 10 grams of starch were boiled for thirty minutes in distilled water. Upon the addition of the minerals of the Czapek's solution (all constituents except sucrose), the volume was made up to one liter and distributed into equal sized test tubes, 10 cc. to each tube. On sterilization, the tubes were inoculated with the different Actinomycetes and both inoculated and uninoculated tubes (for control) were incubated at 25°C. At the end of fourteen to fifteen days, the tubes were taken out of the incubator. The reading was made as follows: One control tube (uninoculated) was kept side by side with the tube containing the organism and the difference in the heights of starch in the two tubes was taken as the diastatic action of the organism. This method has the advantage over the plate method that different layers of the liquid in the tube can be pipetted off and tested for the presence of dextrins and reducing sugars.

The plate method consists in adding 1.2 per cent agar to the liquid medium and pouring 10 to 12 cc. of the sterile liquefied agar into sterile Petri dishes. Upon cooling, these are inoculated and incubated at 25° for fifteen days. At the end of that period, the surfaces of the plates are covered with a solution of iodine and potassium iodide. The clear zone around the growth indicates the diastatic action. The results of both methods are given in table 3. Plate I shows typical colonies with the diastatic zone which can be differentiated from the growth itself.

The two methods correspond fairly well, although they do not go hand in hand, But, with either method, the active diastase production by Actinomycetes is evident. With the exception of *A. asteroides*, all the other organisms hydrolize the starch very rapidly. *A. scabies* produced some hydrolysis as shown by the tube method as well as on the plate.

TABLE 3

The production of diastase by actinomycetes

NAME OF ORGANISM	TUBE METHOD—HEIGHT IN NUMBER OF MILLIMETERS	PLATE METHOD
A. violaceus-ruber	20	++
A. violaceus-caesari	23	+++
A. viridochromogenus	10	+
A. aureus	22	++
A. albus	22	+++
A. exfoliatus	14	++
A. griseus	31	+++
A. poolensis	13	+
A. albosporeus	32	++++
A. lipmanii	32	+++
A. scabies	8	+
A. fradii	25	+++
A. diastaticus	39	++++
A. bobili	24	+++
A. verne	22	++
A. rutgersensis	30	+++
A. asteroides	9	0
120	20	++
168	42	+++
104	16	+
154	20	+++
128	24	+++
215	24	++
205	22	

+ indicates zone 1 to 4 mm. wide; ++ 5 to 10 mm.; +++ 10 to 15 mm.; ++++ above 15 mm.

The process of the transformation of starch through the dextrin stages to reducing sugars is very rapid; in some cases the starch, added to the liquid medium to make up a concentration of 1 per cent, disappeared in seven days, and in many cases in fourteen to fifteen days. The source of nitrogen seems to have

an important bearing upon the hydrolysis of starch by Actinomycetes; Krainsky (1914) has already called attention to this fact, showing that asparagine will stimulate greater diastase activity than KNO_3, NH_4Cl or peptone. The chromogenus types represented in the above table by *A. scabies* and *A. viridochromogenus* have very weak diastatic power. This has already been pointed out before by Krainsky (1914) who stated that the chromogenus species produce very little diastase or none at all.

Four methods were used for the demonstration of cellulose digestion. First the plate method of Kellerman and McBeth described by McBeth (1916); second the method suggested by Krainsky, which consists in placing two pieces of filter paper in Petri dishes, and moistening these with $MgNH_4PO_4$ solution containing 0.1 per cent of K_2HPO_4; third and fourth the addition of filter paper or cellulose suspension to the Czapek's solution without the sucrose as shown above.

The results obtained by the different methods are not concordant. It seems as if some organisms can use the cellulose in one form while others in another. And since all these methods are only poor imitations of natural phenomena and are far from comparing with the natural conditions, under which the organisms live (the digestion of cellulose in the soil is being studied at present), the cellulose decomposition by any one method cannot serve as a positive indication of the ability of a certain organism to attack cellulose. The results are given in table 4.

The cellulose-dissolving power of many Actinomycetes is thus found to be positive by all the methods used. The plate method of Kellerman and McBeth, although well adapted for the study of the growth of the organisms, does not offer any greater advantage than the other methods, since an enzymatic zone was observed for only a few species. The growth of most of the organisms upon this plate was very good, but, as Krainsky (1914) pointed out, this may not be due to the cellulose, but to the agar of the medium. In the growth of the Actinomycetes upon the filter paper, either in the plate or in the solution, free from any other carbohydrate, certain distinctive differences were observed. Certain organisms, such as *A. verne*, *A. exfoliatus*,

and others produced small white colonies right upon the surface of the paper; these separated afterwards and floated through the medium, then falling to the bottom or remaining suspended. Others, such as 120 and *A. violaceus-caesari*, grew upon the paper forming black zones around the colony. Organism 120

TABLE 4

The decomposition of cellulose by actinomycetes

NAME OF ORGANISM	PLATE METHOD (KELLERMAN AND M'BETH)	FILTER PAPER (KRAINSKY)	CELLULOSE SUSPENSION IN SYNTHETIC SOLUTION	PAPER STRIP SYNTHETIC SOLUTION
A. violaceus-ruber	2+	2	3	2
A. violaceus-caesari	1	2	3	2
A. aureus	5	2	0	0
A. albus	3+	5	0	0
A. exfoliatus	3+	1	1	2
A. verne	0		1	3
A. griseus	1	0	1	1
A. poolensis			0	1
A. albosporeus			0	0
A. lipmanii		3	–	0
A. scabies	3		–	2
A. fradii	2	4	0	0
A. diastaticus	1		0	0
A. bobili	3	0	0	4
A. virodochromogenus	2	4	3	1
A. reticuli			3	–
120			1	4
104		0	1	1
168	1	1	1	3
128	1	2	0	1
154	2	1	0	2
205	2		0	2
215	4	5	2	2

0 = no growth; 1 = faint; 2 = fair; 3 = good; 4 = excellent growth; + = production of clearing on cellulose plate.

gave the same characteristic growth upon cellulose as that observed by Krainsky (1914) for his *A. melanosporeus* and *A. melanocyclus* with which 120 is doubtless closely associated.

The utilization of cellulose, where this is the only source of carbon, is also demonstrated by the active reduction of nitrates to nitrites and change in reaction, as shown in table 2.

SUMMARY

1. The utilization of different mono-di- and poly-saccharides, alcohols and salts of organic acids as sources of energy for different Actinomycetes was studied.

2. The order of utilization of the different carbon compounds is as follows:

Starch followed by glucose, lactose, maltose, glycerin, sucrose, cellulose and the organic acids.

3. The best growth is usually accompanied by the greatest reduction of the nitrates of the medium to nitrites and commonly by the greater increase of the pH value of the medium.

4. Few Actinomycetes produce invertase, but even those that do not form this enzyme utilize sucrose to some extent.

5. The production of diastase by Actinomycetes is very prominent and characteristic of the whole group, with very few exceptions. The starch is reduced through the dextrin stage to reducing sugars. In only one or two instances does the reduction seem to stop at the erythrodextrin stage.

6. Some Actinomycetes dissolve cellulose readily.

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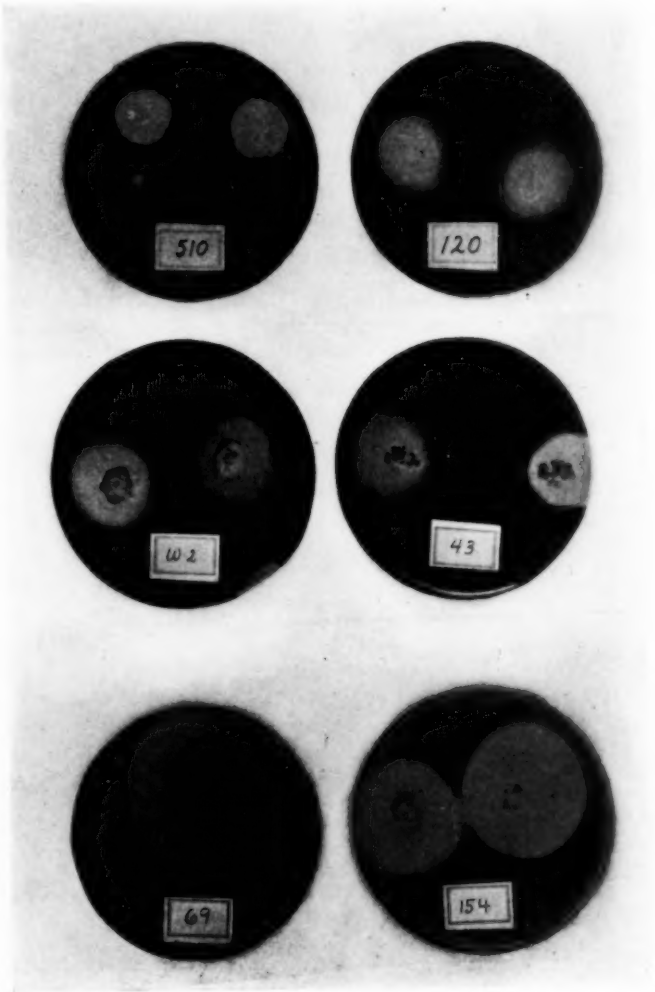
Explanation of Plate 1

Upper photographs (510 and 120), little or no diastase production; the white center indicates the growth of the organisms, while the faint white zone around the growth indicates the diastase production. Middle two photographs (W. 2 and 43), medium diastase production. Lower two photographs (69 and 154), good diastase production. The plates are of regular size (9.25 cm. inside diameter). The cultures were grown for twelve to fourteen days at 25°C., then surface of plate was covered with KI-I solution. The hydrolyzed portion is white, unhydrolyzed blue.



(Waksman: Metabolism of Actinomycetes.)

A SYSTEMATIC STUDY OF THE PROTEUS GROUP OF BACTERIA

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Received for publication March 8, 1919

The term *Proteus* signifies changeability of form, as personified in the Homeric poems in Proteus, "the old man of the sea," who tends the sealflocks of Poseidon and has the gift of endless transformation. The first use of this term in bacteriological nomenclature was made by Hauser (1885) who described under this term three types of organisms which he isolated from putrefied meat. Variations in form and size and in cultural characters were the basis of his classification. Other investigators have since applied the name *Proteus* to specific organisms which they isolated from various sources. Of these organisms some show close similarities and in many instances are identical with the types described by Hauser. Some do not appear, however, to have sufficient common properties to justify inclusion in the *Proteus* group, and attempts to place them here tend to further obscure the poorly defined limits of this group.

The three species which Hauser described under the genus *Proteus* were as follows: *Proteus vulgaris*, which liquefied gelatin and formed zooglea in this medium, and which was very active in its various physiological properties; *Proteus mirabilis* which likewise liquefied gelatin and formed zooglea, but which was less active; and *Proteus zenkeri*, which was unable to liquefy gelatin and which was relatively inactive. The main basis for distinguishing the three species appears to be their action on gelatin.

Babes (1889) isolated an organism from a case of lung gangrene in man which he called *Proteus lethalis*, and another from the

organs of a child which died with symptoms of septicemia, *Proteud septicus*. The same bacterium was later described by Kruse (1896). These organisms appear to be very closely related to one or two of Hauser's types, if indeed they are not identical with them.

Jaeger (1892) isolated a fluorescent organism which, due to some points of similarity with the *Proteus* group, he called *Proteus fluoresecens*. It was described as the causative factor in several cases of Weil's disease. It has since been isolated from similar cases by Bar and Renon (1895), by Conradi and Vogt (1901), and by Bruning (1904). While this organism in a general way bears some resemblance to the *Proteus* group, it is so atypical that its inclusion in this division may be seriously questioned. A more natural grouping would place it in the genus *Pseudomonas*.

Fuller and Johnson (1899) describe two spore-forming organisms as *Proteus*. The property of forming proteus-like colonies on gelatin seems to be their sole basis of classification, hence these organisms may be eliminated from further consideration, especially because no other spore-producing bacteria have been referred to the *Proteus* group.

Prior to the work of Hauser an organism was described by Kurth (1883) which on account of its marked resemblance to Hauser's *Proteus zenkeri* deserves mention here. Kurth's *Bacterium zopfii* was isolated from the intestine of fowls, and has since been observed by others on numerous occasions.

The purpose of the present investigation was to determine the exact relation of the *Proteus* group to other groups of organisms, and to point out more clearly than has been done heretofore the specific properties which serve to distinguish the members of what has so generally been termed the "*Proteus* group."

For this purpose 84 strains were obtained from different sources. Of these 58 were procured from other laboratories, and were labelled as follows:

B. proteus-vulgaris	27
B. proteus-mirabilis	8
B. proteus-zenkeri	4
B. proteus	13
B. zopfii	5
B. proteus-viridis	1

Twenty-six different strains were isolated in this laboratory, of which 25 were of the *Proteus vulgaris* or *Proteus mirabilis*, and one of the *B. zopfii* or *Proteus zenkeri* type.

A morphological and cultural study of the organisms of this collection showed that it could be divided into the three following divisions:

Group I comprising *Proteus vulgaris*, *Proteus mirabilis*, and *Bacillus proteus*.

Group II comprising *Proteus zenkeri* and *Bacterium zopfii*.

Group III comprising *Proteus fluorescens*.

The members of group I are Gram negative and very actively motile, and on agar show a peculiar spreading growth. They usually exert proteolytic action on gelatin and in milk, and to some extent attack carbohydrates, and protein material in general. Furthermore, they grow luxuriantly on all of the ordinary media, and are not limited to any specific temperature range.

The two types which comprise group II are distinctly Gram-positive. They possess no proteolytic action and do not attack carbohydrates; neither do they produce a luxuriant spreading growth on moist agar, as do the members of group I. They develop very poorly in liquid media.

The one available strain of group III differed markedly from the organisms of the other two groups. Fluorescent pigment production, together with its other properties so characteristic of the fluorescent group, should naturally place this strain within the genus *Pseudomonas*.

While Hauser at first described the *Proteus* group as being composed of three distinct species, that is *P. vulgaris*, *P. mirabilis*, and *P. zenkeri*, he later thought that the last two species might be only varieties of *Proteus vulgaris*. His latter conclusion seems to have been accepted by most investigators, though little evi-

dence can be found to substantiate it. Kendall (1916) states "that it is now recognized that cultures of *B. proteus* may gradually lose their gelatin-liquefying power after prolonged cultivation, so that a cultural transition from *B. proteus* to *B. zenkeri* may be observed in the laboratory." While we have observed loss of ability to liquefy gelatin in certain strains, we have never noted other changes in *Proteus vulgaris* which would tend to give it the characters of *Proteus zenkeri*.

In the present study the strains of *Proteus* which were labelled *Proteus vulgaris* and *Proteus mirabilis* when received were found to be practically identical in all of their characters. Both liquefied gelatin with the same rapidity. Although in each species variability in proteolytic action was noted in a few instances, no other changes accompanied the partial or complete loss of gelalatin-liquefying power, and the strains did not in the least assume the characters of Hauser's *Proteus zenkeri*. Thus it appears that the classification of Hauser holds only in so far as the separation of his species *Proteus zenkeri* from the other two is concerned.

Kruse (1896) and Chester (1909) noted a similarity between Hauser's *Proteus zenkeri* and *Bacillus zopfii* of Kurth (1883). We have found the two to be identical, and hence would classify them as one and the same genus under the generic name of Zopfius.

The types which were labelled *Proteus vulgaris*, *Proteus mirabilis* and *Bacillus proteus* have been reduced by us to two species, namely *Proteus vulgaris* and *Proteus mirabilis*, *as B. proteus* is but another name applied to either or both of the others. The *Proteus* group as a whole is sometimes referred to as *Bacillus proteus;* but the use of this name should be discontinued.

In the present investigation the original *Proteus* group of Hauser has been split, therefore, into two distinct genera, namely *Proteus* and *Zopfius*. In the former are included *P. vulgaris* and *P. mirabilis* of Hauser, together with the strains in our collection labelled *Bacillus proteus*, and under the genus *Zopfius*, *Bacterium zopfii* of Kurth and *Proteus zenkeri* of Hauser. The basis for this classification will be brought out further in the data and discussions which follow.

GENUS PROTEUS

This genus may be defined as comprising organisms which in form are small coli-like rods with rounded ends and occurring singly, in pairs or in chains; they are Gram negative, form neither spores not capsules, and are actively motile by means of peritrichiate flagella. Gelatin is usually liquefied rapidly, though this property may be entirely lost. When inoculated into the condensation fluid of slant agar tubes a rapidly spreading growth is produced over the entire surface of the agar. The strains ferment, with acid and gas production, glucose, levulose, galactose, sucrose and glycerol and occasionally maltose. Alkalinity is usually produced in litmus milk, followed by decoloration of the litmus and digestion of the casein. At times there is slight coagulation or precipitation of casein with subsequent re-solution or digestion.

Organisms of this genus are widely distributed in nature, and have been isolated from numerous sources. Their presence in soil appears to depend largely upon recent contamination with animal excreta or putrefactive organic matter of animal origin. Cantu (1911) was able to isolate organisms of this genus from 23 out of 52 samples of garden soil.

Members of this genus are often present in stagnant pools, sluggish streams and other contaminated waters. We have obtained them from stagnant pools, aquaria and street washings. Ward (1899) isolated several strains from the Thames River, and Jordan (1903) from the waters of the upper Mississippi. Horowitz (1916) made several isolations from snow water.

Proteus organisms may be said to be present in practically all sewage, for here there is a constant source of contamination and a favorable medium for development.

The presence of this genus in the intestinal tract of man is by many authorities regarded as an indication of intestinal trouble or some other pathological condition. Ford (1901) claims to have isolated it from various parts of the intestine, but as some of his organisms were fermenters of lactose, there is some doubt as to whether all were *Proteus*. Stewart (1917) be-

lieves that *Proteus* members found in war wounds are of non-fecal origin. In the examination of several thousand samples of feces from dysentery convalescents he found this genus to be a very uncommon inhabitant of the colon of man. It may be obtained from the intestinal tract of lower animals, as for example guinea pigs. Jensen (1903) observed this genus to be present in large numbers in calves affected with a form of dysentery. Its presence in similar conditions in man and animals may in part account for its wide distribution in nature.

The most favorable habitat of the genus *Proteus* is decomposing organic matter of animal origin. In such material it is almost invariably present. Cantu (1911) was able at will to isolate it from putrefied meat, as have many other investigators. Wyss (1898) obtained a strain of *Proteus* from dead fish, and Shrank (1888) from spoiled eggs. Isolations have been made also from human cadavers, where this organism was found in large numbers by Hauser (1885), Hofmeister (1893), Haegler (1892) and Kuhn (1891). We were able to obtain it from meat which had been allowed to undergo decomposition, and from the partly decomposed bodies of dead rabbits and guinea-pigs.

Method of isolation

Until quite recently the usual gelatin and agar plate methods of solation have been employed for this group. As these were very faulty for this type of bacteria, many efforts resulted in failure. The newer methods have rendered valuable service, however. In the present work the procedure of Cantu (1911) was at first adopted. Gelatin tubes are inoculated directly with the material in question. After incubation at 20°C. for several days transfers are made from tubes in which liquefaction has taken place to the condensation fluid of new slant agar tubes. If *Proteus* organisms are present a rapidly spreading growth occurs in twelve to twenty-four hours at 30° to 37°C. This growth is quite characteristic and usually spreads over the entire surface. From the uppermost portion of the surface growth inoculations are made in the condensation fluid of a second agar tube, and the process repeated until a pure culture is obtained.

It soon became apparent in the present investigation that the materials for study could be inoculated directly into the condensation water of the sloped agar tube, and the period in which isolation is effected very much shortened. This modification in no way detracts from the merits of the Cantu procedure. As a rule very little effort is required to effect complete isolation of the *Proteus* genus, owing to its peculiar property of overspreading agar rapidly and leaving associated organisms behind in the condensation fluid. Fresh agar is necessary, however, and the results are greatly facilitated by washing the agar surface with the condensation water just before inoculation.

General characters of the Genus Proteus

The salient features of this genus have already been defined. The following is an elaboration of the different characters, in so far as Journal space will permit.[1]

The individual cells are usually short Coli-like rods with rounded ends, varying in dimensions from 0.4 to 0.6μ by 1.2 to 2.5μ, though occasionally much longer cells are seen. The rods may be grouped singly, in pairs or in short chains. They are actively motile, possessing peritrichous flagella. The unstained cells appear homogeneous in structure. Neither spores nor capsules have been observed. All strains are at all times Gram-negative. Young cultures are readily stained with methylene blue, fuchsin and other common basic dyes.

Members of the *Proteus* genus grow luxuriantly on the usual solid and liquid laboratory media. They are capable of growing within a wide range of temperature, and within reasonable limits development is not materially affected by change in hydrogen ion concentration.

Various ranges of temperature have been reported as most favorable. Hauser (1885) gives 20° to 34°C. as the optimum. Kendall (1916) places it at about 25°. Berthelot (1914), Cantu (1911), and Glenn (1911) grew the organisms successfully at 37°. Levy

[1] For more complete descriptions and discussions the reader is referred to the doctorate thesis (J. J. Wenner) in the Yale University Library.

(1894) showed that the group develops slowly at a temperature as low as 0° and as high as 43° to 45°C. We have invariably obtained maximum growth at 34° to 37°. Good growth was obtained also at 20°, though longer incubation was required, as shown for example in glucose broth culture in which maximum acidity was attained in twenty-four hours at 37°, as against forty-eight hours at 20°, and maximum gas production in twenty-four hours at 37°, as against one hundred and twenty hours at 20°C.

Growth on plain agar

The most characteristic growth of *Proteus* is obtained on slant agar. This was pointed out by Cantu (1911) when he showed that inoculation in the condensation fluid of fresh sloped agar resulted in a uniform growth over the entire surface. This growth may be homogeneous, or of a more or less peculiarly modeled character. It is of a butyrous consistency. If the surface of the inoculated agar is dry a streak inoculation results in a pronounced growth which spreads very irregularly, with a more or less lacerated margin. The extent of the spreading depends on the amount of moisture on the agar.

Colony growth on plate agar may be at times characteristic, that is, of ameboid appearance, or in the form of large colonies which are more or less rosette-like, with very irregular borders. Again, the colonies may be small and with entire margin, resembling those of *B. coli*.

Action on gelatin

Much interest has centered around the property of gelatin liquefaction of this genus. Hauser in his original work laid special emphasis on it and used it as the chief basis for his distinction of types. Since then marked variations in individual strains have been observed by different investigators. Smith (1894) was able by selection to transform a liquefying *Proteus vulgaris* into a non-liquefying strain. Herter and Broeck (1911) showed that a liquefying strain of *Proteus vulgaris* which had

lost its liquefying properties, but remained typical in other respects, could have the lost function restored by passage through a mouse.

Of the 73 strains studied in this investigation, 3 lost the property of liquefying gelatin while in other ways they remained typical. Two of these organisms were old laboratory strains labeled *Proteus vulgaris;* the third was isolated from putrefying meat. We were unable to restore the liquefying function by a single passage of one of these strains through a white rat.

On gelatin plates of *Proteus* small colonies are noticeable in eighteen to twenty-four hours. They show an entire margin at first, but as they increase in size irregular spreading may occur. Liquefaction soon takes place and the colonies assume a dewdrop appearance. Radiating filaments extend from the liquefied zone into the surrounding gelatin. The colonies increase in size until the entire plate is liquefied. Hauser employed 5 per cent gelatin, and describes the occurrence of wandering ameboid colonies, that is, irregular masses of cells which constantly underwent changes in form and position, and sometimes separated from the mother colony. In order to obtain colonies that are at all characteristic gelatin of rather soft consistency is required. On the usual 10 per cent gelatin the colonies are often entire and without distinguishing marks.

In gelatin stab cultures liquefaction begins at the surface, soon becomes stratiform and eventually involves the entire tube. The rate depends on the temperature and an abundance of free oxygen. Liquefaction may be completely inhibited by a layer of oil over the surface of the gelatin. The oxygen is essential in the production of the proteolytic enzyme.

Growth in bouillion

Marked turbidity is rapidly produced, reaching its maximum in from three to five days at 30 to 37°C. Young cultures usually show no surface film, while older tubes gradually develop a thin brittle pellicle which is easily broken up. As broth cultures present few if any features which are characteristic and of special

interest no further comments are necessary. Nitrite is formed in nitrate broth.

Action of the genus Proteus in milk

As a rule vigorous development occurs in milk, and a marked change may be brought about in the appearance of this medium in twenty-four to thirty-six hours at 37°C., the litmus being reduced and coagulation or digestion of the casein taking place. The rate of transformation varies with different strains, some of them completely digesting the casein in three to five days. On the other hand, other strains appear to have lost this proteolytic power completely. The usual change observed in this study of 73 strains was an initial alkalinity which gradually became more intense and was followed by decolorization of the litmus and digestion of the casein. Some strains (3) showed slight acid production at first. Casein was digested by 69 strains.

The ability of organisms to digest casein was demonstrated definitely by growing them in a medium containing, besides 0.5 per cent meat extract and 0.5 per cent sodium chloride, 0.2 per cent of purified casein, and observing the loss of the protein by means of the biuret method of Vernon (1903), or by precipitation with acetic acid.

Action on carbohydrates, glycerol, etc.

Fermentation is limited to glucose, levulose, galactose, sucrose maltose and glycerol. The glucose, levulose, galactose and glycerol were attacked more or less uniformly by all strains, sucrose readily by some and slowly by others, and maltose only by some of the strains. Fermentation in all cases comprises both acid and gas production. The medium employed in the fermentation experiments was plain sugar-free broth to which 1 per cent of the carbohydrate in question was added. Other agents used were lactose, inulin, dulcitol, mannitol, sorbitol, salicin, raffinose, arabinose, adonitol, dextrin and starch. The results with these were negative.

Glucose

This is one of the most favorable sources of energy for the organisms of the *Proteus* genus. Its presence in a medium considerably hastens growth. From 25 to 30 per cent of gas, and from 2.5 to 3 per cent of acid in terms of N/20, with phenolphthalein as an indicator, are produced. These results agree with those of other investigators.

Sucrose

Smith (1893) was the first to show that the action of this group on sucrose was practically the same as on glucose. Similar results have been obtained since by other investigators, though Glenn (1911) found several indifferent strains among his stock cultures, and Horowitz (1916) reports a positive reaction in only 7 out of a total of 24 strains.

In the present investigation a variation in the action of *Proteus* on this sugar was noted, some strains producing the maximum amounts of acid and gas in twenty-four to seventy-two hours, while others required twelve to fifteen days. The delayed action of the latter (8 or 9 days) was at first overlooked, but it was observed that when the period of delayed action was passed the fermentation was as pronounced as with the strains which attacked the sucrose immediately. Of the 73 strains studied, 25 showed an immediate, and 48 a delayed action. In correlating these results with the action on other carbohydrates, it soon became apparent that the strains which fermented sucrose readily also fermented maltose, while those which showed delayed action on sucrose did not attack the maltose.

Maltose

Maltose appears to be the only carbohydrate that is of any value as a means of subdividing the *Proteus* group. Berthelot (1914) noted a variation in the action of different strains on this sugar. Horowitz (1916) found that 23 out of 24 strains fermented it with the production of acid and gas; and Stewart (1917) observed 2 out of 29 having this property. Of the 73 strains in the present collection, 25 showed distinct acid and gas production. No

delayed action on the sugar could be detected, as in the case of sucrose.

Galactose, levulose and glycerol

While these agents are fermented by this group the action is not so marked and does not occur as readily as with the sugars just mentioned. The amount of gas produced may vary from a mere bubble to 20 per cent, and the acid from 1 to 2 cc.

Lactose

Conflicting results have been reported. While most investigators have claimed that lactose is not attacked by the *Proteus* group, others have observed fermentation with acid and gas production. In the light of our own experiments these conflicting results may be explained by the presence of an available carbohydrate as an impurity in the lactose. When absolutely pure lactose was employed no fermentation could be detected under either aerobic or anaerobic conditions.

Growth on potato

On cooked potato prepared in the usual way very luxuriant growth is produced. It appears within twenty-four hours along the line of inoculation and gradually spreads over the surface irregularly. It is of a butyrous consistency and of a dirty brown color which quickly diffuses through the potato. A characteristic fish brine odor is produced in this medium.

Browning of lead acetate medium

All of the 73 strains of *Proteus* used in this investigation caused a distinct browning of a medium consisting of 0.5 per cent nitrate agar, 0.05 to 0.1 per cent lead acetate, and 0.2 per cent glucose.

Hemolytic action

This genus is unable to hemolyze red blood cells. Different strains were tested both in suspensions of washed erythrocytes and on plates of sterile blood agar.

Growth in synthetic media

Development in Uschinsky and similar synthetic media is limited. It becomes more marked, however, when glucose is substituted for glycerol in the medium. In the phthalate medium of Clark and Lubs (1917) growth is likewise limited.

Chromogenesis

With the exception of a few investigators (Ward, 1899, and Jordan, 1903) the *Proteus* group is considered as non-pigment producing. In the present work no color production was noted in any of the media except the brownish growth on potato and the gradual browning of the potato itself.

Changes in hydrogen ion concentration

In plain bouillon prepared from Liebig's beef extract and Witte's peptone no change in titratable acidity was noted, while hydrogen ion determination by the newer colorimetric method showed slight alkali production. In plain bouillon containing an available carbohydrate sufficient acid is produced to bring the H ion concentration to about 5 on the colorimetric scale. Similar results were obtained in the special peptone medium of Clark and Lubs (1917). Little acid production occurs, however, in their phthalate medium owing to the limited growth of the organisms.

Indol production

Indol production by this genus has been pointed out by many investigators. Variations in this property have been noticed also. Steensma (1906) studied several strains which failed to produce indol. Van Loghem and Van Loghem-Pouw (1912) made two subdivisions out of the strains under observation, namely *B. proteus-anindologenes* and *B. proteus-indologenes*. Berthelot (1914) found that 24 out of a total of 61 strains formed indol; Horowitz (1916), 7 out of 24; and Stewart (1917) 1 out of his collection of 29.

In the present work results were obtained which varied with the methods employed. Dunham's solution, sugar-free broth, and a 1 per cent solution of predigested casein were used. Both the Salkowski and the Ehrlich aldehyde method were employed. Of the 73 strains all gave a positive reaction with the sulphuric acid and nitrite test of Salkowski, while 46 gave a reddish color on the addition of the acid alone. With the Ehrlich method 33 of the 73 strains gave a strongly positive, 36 a slightly positive and 4 a negative reaction. These variations were obtained in each of the 3 media.

Hydrogen sulphide and mercaptan

All of the strains formed hydrogen sulphide in appreciable amounts. On the other hand, little if any mercaptan could be detected. Mercaptan production has been the subject of investigation on previous occasions. It has been assumed by many that this is a common product of *Proteus*, because this genus is so constantly present in organic matter undergoing putrefactive decomposition, though it is not itself a strictly putrefactive organism. Rettger (1906) found no mercaptan in anaerobic cultures of *Proteus vulgaris* in egg-meat mixture. Herter and Broeck (1911) also were unable to detect it in plain bouillon cultures, even when cystin was added. Ward (1916) claims, however, that he obtained marked mercaptan production with 4 different strains which he grew in plain bouillon.

Nine strains were tested for the property of mercaptan production by the method formerly employed by Rettger, and involving the use of isatin-sulphuric acid and of mercuric cyanide. In some instances a slight change in the color of the test solutions could be detected, but as control flasks gave a similar change in color, little, if indeed any, mercaptan was present in the culture flasks. Contamination of such flasks with a putrefactive anaerobe, however, soon resulted in abundant mercaptan production.

Putrefaction

The experiments of Hauser (1885), Emmerling (1896) and others, demonstrating putrefactive changes in what appeared

to be pure cultures of *Proteus* organisms, as well as the frequent assertions that members of the *Proteus* group are always present in organic matter that is undergoing putrefaction, has led to the assumption that this group has distinct putrefactive properties. Rettger and Newell (1912) have shown more recently that no decomposition of protein material takes place under anaerobic conditions when pure cultures of *Proteus* are used. Similar experiments were conducted in the present investigation, and the results of Rettger and Newell corroborated. No changes in the character of protein material could be brought about by pure cultures of *Proteus vulgaris* in the absence of atmospheric oxygen, whether in milk, egg-meat mixture, or other protein-containing medium. There was no reduction in the volume of the solid matter in the egg-meat medium, nor could any of the foul smelling products of putrefaction be detected. Furthermore, there was very little, if indeed any mercaptan present in the medium. Under aerobic conditions, however, the ordinary non-putrefactive products of protein decomposition are produced.

Agglutination

Several attempts have been made in the past to employ agglutination as a basis for subdividing the *Proteus* group. Cantu (1911) showed that the blood serum of animals which had been injected with heated suspensions of these organisms had agglutinating properties which, barring some exceptions, were specific for the strains injected. He concluded that this method can not be employed for subdividing different strains. Van Loghem and Van Loghem-Pouw (1912) claimed that indol-producing strains could be distinguished from those which do not form indol, by their agglutination properties. Horowitz (1916) obtained cross agglutinations among homologous strains, and thereby was able to split the *Proteus* group into 5 subdivisions, the members of each having specific properties, as regarded indol production and carbohydrate fermentation.

In the present work several rabbits were immunized against specific strains of *Proteus vulgaris* and *Proteus mirabilis*. Killed

suspensions were injected at first, followed by at least one or two suspensions of living organisms grown on slant agar and washed off with saline solution. After each injection the animals showed some loss in weight which was very soon regained. At the site of inoculation a large abscess was formed which disappeared only after several months. The production of agglutinins could be demonstrated very soon after the first injection. After the last injection agglutination in as high as 1:100,000 dilution took place.

The different strains of *Proteus* were tested by the macroscopic method in dilutions of 1:50, 1:100, 1:500, 1:1000, and 1:5000. Seven different sera were prepared with as many strains of *Proteus*. Table 1 shows the number of strains agglutinated by each serum. With one exception, all of the sera agglutinated other strains besides those employed in their preparation. Some strains were agglutinated by more than one serum. Nineteen of the strains used in the agglutination tests failed to be agglutinated by any of the sera. It would appear, on the whole, that the *Proteus* group is more or less heterogeneous, like the *Streptococcus* and *B. dysenteriae* group. While the agglutination method may be of some value, in identifying members of the *Proteus* group, negative results do not necessarily exclude an organism from this group.

Pathogenicity

The occurrence of the genus *Proteus*, either in pure culture or in association with other organisms, in pathological conditions, has been reported by various investigators. Foa and Bonome (1889) isolated it from a case of volvulus, Schnitzler (1890) and Krogius (1890) from cases of cystitis, Flexner (1893) from a patient having peritonitis, and Reed (1894) in croupous pneumonia, associated with a pneumococcus. Booker (1897) and Metchnikoff (1909) made *Proteus* isolations from cases of diarrhea in children, Vincent (1909) from typhoid fever patients, and Horowitz (1916) from persons suffering with gastro-enteritis. Larson and Bell (1915) recovered *Proteus* organisms from a

laparotomy wound, infected eye and finger, from the heart's blood of a fatal case of peritonitis, and from one of gangrene of the lung. Ward (1916) obtained it from supposed diphtheria subjects and from typical cases of atrophic rhinitis.

Dudgeon, Gardner and Bantree (1915) found typical *Proteus* in 5 per cent and atypical *Proteus* in 2 per cent of a total of 100 cases of war wounds. Goadby (1916) encountered *Proteus* in 47 per cent of the 200 wounds studied bacteriologically. Distaso

TABLE 1

Number of strains agglutinated by each serum or combination of sera

NUMBER OF STRAINS	SERA*						
	A	B	C	D	E	F	G
1	+	0	0	0	0	0	0
1	+	+	0	0	0	0	0
1	0	0	0	0	0	+	0
8	+	+	0	0	+	0	0
3	0	+	0	+	0	0	0
1	0	+	+	+	0	+	0
1	0	0	+	+	0	0	0
23	0	0	+	+	0	+	0
11	0	0	0	+	0	0	0
3	0	0	0	+	+	0	0
1	0	0	0	0	0	0	+
19	0	0	0	0	0	0	0
Total, 73	10	13	25	42	11	25	1

* Sera A, B, C, D, E and F were prepared with stock strains of which the first five were labeled *Proteus vulgaris* Hauser and the last *Proteus mirabilis* Hauser. Serum G was prepared with a strain isolated by us from putrefied meat.

(1916) found coliform organisms including *Proteus* predominating in the first stages of wound infection. He suggests the use of *Proteus* vaccine along with others in the treatment of war wounds. Stewart (1917) isolated 29 strains of the *Proteus* genus from infected war wounds, or a case rate of 24 per cent.

While this genus is ordinarily regarded as non-pathogenic, there is ample evidence to show that it may assume a pathogenic rôle, and thus occupy a position analogous to the pyogenic micrococci. The pathogenicity varies in experimental animals,

some strains causing death in 16 to 24 hours, while others cause no apparent ill effects. For example, Kühnau (1897) found that strains from several cases of diphtheria, and Larson and Bell (1915) that some strains of *Proteus* obtained from human lesions, were decidedly pathogenic for rabbits, guinea-pigs and rats. In general the virulence of a strain is shown by the production of local pathological conditions or by symptoms of intoxication.

In the present investigation both virulent and non-virulent strains were met with. One of the most pathogenic was an old stock culture of unknown origin, this indicating that virulence may be mantained indefinitely.

Hauser, in his original work on the *Proteus* group, found that boullion and gelatin cultures were toxic and produced fatal results when injected into animals. Other investigators have obtained similar results. The nature of the toxicity is not known, although the effects are apparently those of real toxemia.

The toxicity of several strains of the *Proteus* genus was demonstrated by the writers by injecting 2 cc. of saline suspensions from 24-hour agar cultures. Subcutaneous injections in rabbits produced abscesses and inflammatory conditions which lasted several months, usually accompanied by loss of weight, weakness and lessened appetite. In white rats the results varied with the strains, some causing symptoms of toxemia and killing the animals in eighteen to twenty-four hours, when injected by the subcutaneous route. Others caused no apparent ill effects even when the injections were intraperitoneal. One strain caused the formation of an abscess in one rabbit, and definite symptoms of toxemia and death in another. In the fatal cases the organisms could be isolated from the blood and internal organs. Killed suspensions when injected into rabbits caused definite lesions at the site of inoculation.

Classification of species

Since Hauser's classification several investigators have attempted to group the various strains of the genus *Proteus* on properties other than gelatin liquefaction. Ford (1901) defines

the *Proteus* group as consisting of alkali-producing non-chromogenic, non-sporing bacilli capable of liquefying gelatin, casein and blood serum. He made a further division, on the basis of motility and carbohydrate fermentation, into six varieties, two of which fermented lactose.

In his study of bacteria found in river water, Jordan (1903) divided the *Proteus* group into two subdivisions, namely the *Proteus vulgaris* type and *Proteus* varieties. The first of these he described as always fermenting glucose and sucrose, with gas production, but never lactose; liquefying gelatin, casein and blood serum, and curdling milk, with acid production. The second subdivision differed from the first mainly in its proteolytic action. Cantu (1911) in a study of 184 strains isolated from various sources was unable to subdivide them. Van Loghem and Van Loghem-Pouw (1912) were able to divide a series of strains obtained mostly from intestinal contents into two groups on the basis of their indol-producing function. The strains belonging to one or the other group were similar in their agglutinating properties. Horowitz (1916) divided 24 strains into 5 subgroups, on the basis of agglutination. Stewart (1917) found 27 strains isolated from war wounds to differ in their action on maltose and litmus, and in their motility and idol production.

The present investigation has shown that attempts of others to divide the *Proteus* group into two or more subdivisions are unsound. The classification of Hauser on the basis of gelatin liquefaction is of little value, since this property is too irregular and inconsistent. In their agglutination power the members of the *Proteus* genus are heterogeneous in character, so that no distinct separation into species is possible on this basis. Indol production is also very unsatisfactory as a distinguishing character. The only property which appears to us to be of value in making subdivisions of the genus *Proteus* is that of carbohydrate fermentation. Several investigators have noted a difference in the action of individual strains on maltose. Of the 73 strains employed by us 25 fermented this sugar, while the remaining 48 failed to do so. A definite correlation existed between the property of attacking maltose and

the rapidity with which sucrose was fermented with gas production. All of the strains which fermented maltose, with both acid and gas production, also fermented sucrose readily, while all of those which failed to attack maltose showed a delayed action on sucrose and brought about visible acid and gas production only after the expiration of eight to nine days.

While no other property could be correlated with this action on the sugar, it lends itself as a definite basis for dividing the *Proteus* genus into two species, the one fermenting maltose with acid and gas production, and the other being unable to attack this disaccharide. For the former the name *Proteus vulgaris* may be retained, while for the other *Proteus mirabilis* is here suggested. By retaining these names the nomenclature would be simplified. The differentiating characters of Hauser must be set aside, however, in order to avoid confusion.

GENUS ZOPFIUS

Under this genus the types formerly known as *B. zopfii* and *Proteus zenkeri* will be described. Very few strains of these organisms are kept in stock, as only 4 strains of *Proteus zenkeri* and 5 strains of *B. zopfii* could be obtained by a canvass of 40 bacteriological laboratories. To these 9 strains one was added which we were able to isolate from putrefied meat. All of these strains were practically identical.

The individual cells are rod-shaped, usually about 0.8μ by 3.5μ in size, have somewhat rounded ends, and in young cultures occur in long evenly-curved chains. They stain well and are Gram-positive. The organisms are motile, having peritrichous flagella, but do not form spores or capsules. They are facultative anaerobes and grow well on the surface or directly beneath the surface of agar and gelatin. In gelatin stab tubes an arborescent growth results which is most luxuriant at the top of the stab. In plain bouillon growth is slow and moderate, while in litmus milk it is very scant and produces no visible change. Gelatin is not liquefied, and none of the carbohydrates are attacked. On potato the growth is moderate with subsequent darkening of

the medium. The most favorable temperature for this genus is about 25°C. Good growth also occurs at 20° and at 30°, while at 37°C. the growth is very poor. No distinguishable odor was noted on any of the cultures. Hydrogen sulfide was not produced and growth in egg-meat medium was poor, resulting in no visible changes. On slant-agar and in agar and gelatin plates a more or less characteristic spider web growth often develops, but inoculations in the condensation water of slant agar do not cause a spreading over the surface. A division of the various strains into species did not seem possible on account of the few differentiating properties of these organisms.

SUMMARY AND CONCLUSIONS

The *Proteus* group has been known to include various types of organisms some of which have few common properties.

The types *Proteus vulgaris* Hauser, *Proteus mirabilis* Hauser, and *B. proteus* are, with a few exceptions, identical. The genus *Proteus* should be limited to organisms of this group.

Proteus zenkeri is identical with *B. zopfii* and therefore should not be grouped with the *Proteus* genus but rather with *B. zopfii*, the organisms of this type forming a genus to be known as *Zopfius*.

The organism *Proteus fluorescens* Jaeger does not resemble the *Proteus* genus, but rather the fluorescent group (genus *Pseudomonas*), and should not be known by the name *Proteus*.

The *Proteus* genus comprises a large group of organisms which can be subdivided on the basis of their action on maltose into two distinct species. For the species fermenting this sugar the name *Proteus vulgaris* is suggested, and for the species failing to attack it the name *Proteus mirabilis*. The genus cannot be subdivided satisfactorily on the basis of proteolytic action, indol production, or agglutinating properties.

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ON THE BACTERIOLOGY OF DYSENTERY IN NORWAY

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Received for publication March 7, 1919

After the first description of the dysentery bacillus by Shiga and the subsequent detection of the socalled mannit-fermenting strains (pseudo-dysentery bacilli, atoxic dysentery baccilli) by Flexner, Strong and Hiss and Russell the knowledge of these bacilli remained practically unaltered for a rather long time. It is only the last few years that have brought some new development to the study of bacillary dysentery.

Of very great importance in this new development is the description by Sonne in Demark of a hitherto unknown type of the dysentery bacillus. This type, which Sonne calls group III, had been seen by some authors before him (Baerthlein, Losener and others). But these authors had considered the microbe as an inagglutinable type of the Flexner bacillus and had not taken any interest in the study of it. Sonne, however, showed that this microbe serologically (by agglutination) was very specific, and absolutely different from the other dysentery bacilli. He further showed, that this microbe was the main cause of dysentery in the material studied by him in Copenhagen.[1]

After Sonne the microbe was isolated in France by D'Herelle who did not know of the description by Sonne. Further the microbe has been found by the author of this paper in a quantity of cases of dysentery in Norway, and lastly by Oehnell in Sweden.

[1]Extract from a larger work "Om Dysenteri i Norge" (On Dysentery in Norway), Medicinsk Revue, Bergen, 1917, and from a smaller one "Yderligere bidrag til kjendskapet til dysenteribaciller av gruppe III" (Further contributions to the knowledge of dysentery bacilli of group III), Medicinske Revue, juli, 1918.

Another very important result of the study of dysentery during the last years is the statement, that the old types Y, Flexner and Strong cannot any longer be considered as different types. They must be united into one main group of bacilli, that show some individual variation in their fermenting of sugar broth. This statement was first made by Sonne, who arranged those of his types, that corresponded to the old mannit-fermenting types, into two serologically slightly different groups. He considers these groups as closely connected and is of the opinion that they will probably soon be united into one main group. He names them atoxic groups I and II.

Many other authors (Arnheim, Hehewerth, Chick, Pribram and others) are of the same opinion as Sonne and even claim the serological identity of all the mannit-fermenters, or only find small individual variations insufficient to form a just basis of grouping. They also find the cultural characters inadequate for dividing the bacilli into well characterized and different types. It must, however, be mentioned that all these authors except Sonne deal only with the types Y, Flexner and Strong, not with Sonne's group III. Thus it is at the time most convenient to group the mannit-fermenting dysentery bacilli into two main groups, one containing the old types Y, Flexner and Strong, the other group III of Sonne.

The material for this investigation has been stools from patients suffering from typical dysentery or acute diarrhoea, all from Bergen, or neighboring towns or rural districts on the west coast of Norway. Sixty-five strains of dysentery bacilli have been isolated. About 50 of these have been thoroughly studied as to their serological and cultural characters, while the remaining 15 only have been put through the tests necessary to state their identity and classify them in the right group.

The technique of the examination has been to plate out on litmus-lactose-agar shreds of mucus from the stools, which have been washed several times in saline solution. The blue colonies on the plates are tested with highly agglutinating sera from rabbits, immunized with the various strains belonging to the mannit-fermenters as well as with a strain of the non-man-

nit-fermenting type of Shiga. After this the colonies are cultivated and tested as to their fermentation of sugar (mannit, maltose, glucose and sucrose), and the non-motility of the bacilli is established in a young culture in beef broth.

I. SEROLOGICAL CHARACTERS

1. Agglutination

The strains first isolated were tested with sera partly from Germany, partly from Denmark. After this the strains were inoculated into rabbits, and there were produced several sera for our Norwegian strains. As the Danish mode of grouping has been used in the classifying of the strains, this will be retained here as well, although it will be shown, that the Danish groups I and II in reality are so closely connected, that they may be considered as one group.

The sera used fall into three different groups:

A. Serum produced with a Shiga strain.

B. Four sera produced with two strains belonging to each of Sonne's groups I and II.

C. Two sera produced with strains belonging to Sonne's group III. With these sera cross agglutination with homologous and heterologous strains has been carried out as shown in table 1. The results have been obtained by naked eye examination after two hours in the incubator at 37°C. As antigen is used an emulsion of living bacilli in saline solution after twenty-four hours of growth on agar.

From table 1 it will be seen that the strains isolated in this material fall into two sharply divided groups. The first one is named B, the second C. The letter A is reserved for the Shiga strain. These letters are not proposed as group names, but used here to avoid confusion with the Danish groups I, II and III. From table 1 it will further be seen, that our group B includes specimens of strains from the Danish group I as well as from group II, while our group C corresponds to the Danish group III.

The groups A, B and C differ distinctly from each other, but so that there is a very slight connection between A and B, and no

TABLE 1

Agglutination in immune sera from rabbits (the figures signify dilution of serum)

TESTED WITH			SERUM FROM RABBITS IMMUNIZED AGAINST			
			A	B		C
Groups		Strains	Dys. Shiga	Danish group I	Danish group II	Group III
			Titer 1: 1600	O 36 Titer 1: 6400	F 31 Titer 1: 3200	F 79 Titer 1: 400
A		Dys. Shiga	1600	400	400	0
B	Danish group I	0. 36	100	640	3200	0
		F. 11	100	3200	1600	0
		F. 13	100	3200	3200	0
		F. 16	100	3200	3200	0
		F. 51	100	3200	1600	0
		F. 52	100	6400	1600	0
		F. 55	100	3200	1600	0
		F. 60	200	6400	3200	0
		F. 61	100	6400	3200	0
		F. F2	100	6400	3200	0
		F. F3	100	6400	1600	0
		F. F5	100	3200	3200	0
		F. F8	100	3200	3200	0
		F. 90	400	3200	3200	0
		F.10F	100	6400	1600	0
	Danish group II	F. 29	200	800	3200	0
		F. 30	200	1600	3200	0
		F. 31	200	1600	3200	0
		F. 5F	200	1600	1600	0
		F. 92	400	200	3200	0
		F. 99	0	200	3200	0
		F. 103	100	400	3200	0
		F. 108	?	640	6400	0
		F. 109	?	640	6400	0
C	Group III	F. 40	0	0	0	400
		F. 41	0	0	0	400
		F. F1	0	0	0	400
		F. F6	0	0	0	800
		F.FF	0	0	0	400
		F. F9	0	0	0	400
		F. 86	0	0	0	400
		F. 9F	0	0	0	400
		I	0	0	0	400
		II	0	0	0	400
		III	0	0	0	400
		IV	0	0	0	400
		V	0	0	0	400
		VI	0	0	0	400
		VII	0	0	0	400
		IX	0	0	0	400

connection whatever between these groups and C. As to the relationship between the Danish groups I and II, the table shows that none of the strains in group I are inagglutinable in the sera from group II and vice versa. The strains of group II, however, show a lower agglutinability in the sera from group I than do the homologous strains of group I. On the other hand, the strains from group I are agglutinated to the same titre by serum from group II as the strains from this group themselves.

Consequently there is some difference in the results of the agglutination between the Danish groups I and II. But this difference does not seem large enough to justify their separation into different groups. We have therefore considered it convenient to place them in one main group.

As to the group C (Danish group III) the table will show the absolutely isolated position of this microbe in the scheme of dysentery bacilli. It will also show the low titre of the agglutination of the strains, belonging to this group compared to that of the other strains. Further the agglutination itself is remarkable in this group. There are never to be observed coarse flocculi in the emulsion here such as are seen in the other groups, but always rather fine flocks.

Out of 65 strains of bacilli isolated in this material 40 belonged to group B, 25 to group C. This is not to be explained on the ground that group C consists of rarer microbes than group B. But while the latter group B as a rule gives rise to typical dysentery and attracts notice and therefore naturally contributes largely to this material, the members of group C often cause only a mild diarrhoea, that is not thought serious enough to be treated by a physician and accordingly is not examined.

The examination of agglutination reactions in the sera of dysentery patients cannot be said to be of any great importance in the diagnosis of the disease as the stools render a better material for the examination at an earlier period in the disease than the serum.

Twenty-two different sera from patients have come to examination. Of these 18 were suffering from dysentery at the

time of examination, while 4 had passed through the disease earlier.

TABLE 2

Agglutination in sera from dysentery patients (the figures signify dilution of serum.)

SERUM FROM CASE NUMBER	CASE BELONGS TO GROUP		DAYS AFTER BEGINNING OF DISEASE	AGGLUTINATION WITH MICROBES FROM			
				Own strain	Group B		Group C
					Danish group I	Danish group II	Danish group III
52	B	Danish Group I	7	800	200		0
			28	200			
60			3	40	40	40	0
			10	200	200	200	0
62			14	640	640	40	0
75			10	80	80	40	0
78			18	320	320	40	0
90			6		80	160	0
			15	320	320	320	0
99		Danish group II	7		160	160	0
			16	200	200	200	0
			45	320	200	200	0
41	C	Danish group III	5	20	10	10	20
			9	40	10	10	40
71			10	0	0	0	0
86			10	40			40
I			10	40			Not tried
II			10	20			Not tried
III			10	80			Not tried
IV			10	40			Not tried
V			10	40			40
VI			10	80			80
VII			10	10			10
IX			10	40			40

As shown in table 2 all the patients suffering from infections due to group B gave positive agglutination tests at the end of the first week of the disease and later on. The titre did not seem to increase much in the course of disease. Some of the sera only

gave a positive reaction with the most closely connected strain, a fact that shows the necessity of testing the sera from dysentery patients with several strains before calling the test negative.

Eleven sera from patients suffering from infections with strains belonging to group C have been examined. One of these sera showed an absolute negative reaction, while the others gave positive tests in a dilution of serum from 1:10 to 1:80. The same fine flocculation was seen here as described above.

Tested in normal human sera the strains of group B as a rule showed agglutination up to the dilution 1:40, while group C never showed the slightest sign of agglutination in the same sera. It is therefore obvious that even a low titre of agglutination of group C in a certain serum renders it very likely that the patient, from whom the serum is derived, is suffering from an infection due to a member of this group. On the other hand the titre of a member of group B must be higher than 1:40 to allow any conclusions as to infection.

The titre 1:40 is a good deal lower than that whfch most other authors have found. Thus Sonne found titres in normal sera up to 1:250, Loewenthal up to 1:100, while Frankel only reports a titre of 1:40.

2. Absorption of complement with immune sera from rabbits

The test of Bordet-Gengou has not been very much used in the study of the dysentery bacilli, and the few contributions to the knowledge about it are contradictory. When the previous workers on this subject (Dopter, Haendel, Schroeter and Gutjahr, Amako and Kojma and others) disagree as to the value of the test, the cause may be sought either in inexactness of the reaction itself or in the technique of the worker. It was therefore thought to be of some interest to test this reaction as carefully as possible and with the same sera and strains used in the agglutination test.

As to the antigen, the earlier workers have mostly used an antiformin extract of the bacilli and claimed this to be better than the emulsion of bacilli. As regards the reaction in typhoid

it is proved by Brekke that the emulsion is quite as useful as the extract. But it is questionable, whether the destroying of the bacilli by antiformin and the following neutralization by acid is not apt to rob the bacilli of a good deal of their specificity, at any rate to a greater degree than the mere killing by heat. Accordingly an emulsion of bacilli in saline solution, heated for one hour to 60°C. has been used as antigen. The dose used has been one-twentieth of an agar slope culture (0.5 cc. of a suspension made in 10 cc. of saline solution).

As amboceptor have been used the same sera, used in the agglutination test. The sera have been titrated from 0.1 down to 0.00005 by halving each dose (0.1, 0.05, 0.025, etc.)

The dose of complement necessary for the hemolysis is in each case titrated out before the test itself. For the absorption test the dose is doubled and the solutions of dysentery serum and the emulsion of antigen, 0.5 cc. volume of each, are added.

The tubes are now placed in a waterbath at 37°C. for one and one-half hours, when 0.5 cc. of an emulsion of 5 per cent sheep blood corpuscles and the same volume of hemolytic rabbit-serum (1: 100) are added to each tube. After another one-half hour in the waterbath, the tubes are taken out and placed in the cold to sediment until the next day, when the titres of absorption are noted.

Using this method the absorption of complement has proved very valuable and specific. The results correspond very closely to those of the agglutination test. The strains isolated in this material were divided into the two main groups B and C, already mentioned. These groups were absolutely different in their reactions, while group B showed some, but not much relation to group A (the Shiga strain). Thus the absorption of complement separated the dysentery bacilli into the same three groups A, B and C that had already been identified with the agglutination test.

The results are put down in table 3.

TABLE 3

Absorption of complement in immune sera from rabbits (the figures signify the smallest dose of serum that gave positive reaction).

TESTED WITH			SERUM FROM NORMAL RABBIT	SERUM FROM RABBITS IMMUNIZED AGAINST			
				A	B		C
Groups		Strains		Dys. shiga	Danish group I O36	Danish group II F31	Group III F41
A		Dys. Shiga	+	0.0002	0.025	0.0125	+
B	Danish group I	O36	+	0.1	0.0008	0.0016	+
		F52	+	+	0.0032	0.0125	0.1
	Danish group II	F29	+	0.0125	0.0032	0.0002	0.1
		F31	+	0.0063	0.0032	0.0002	+
		F57	+	0.0125		0.0002	0.1
C	Group III	F40	+	+	+	+	0.0032
		F41	+	+	0.1	+	0.0016

3. The bactericidal effect of normal sera and of immunesera upon the dysentery bacilli

The bactericidal method used in this investigation is that described by Neisser and Wechsberg, slightly modified by Haaland and Brekke. It is necessary to state the main lines of the reaction.

As immunesera have been used the same sera as in the agglutination and complement absorption tests. As complement, sera from normal guinea-pigs have been used. Before the reaction itself can be carried out, the complement must be titrated to find its own bactericidal effect upon the bacteria in question. This effect is called the total bactericidal effect. It is necessary to find this effect, as the dose used in the test itself must be lower than the lowest dose of complement that has any bactericidal effect itself without immuneserum. Simultaneously a test is carried out to find the activating power of the complement upon the immuneserum. The dose of complement employed in the bactericidal reaction must be smaller than the smallest dose that

gives a total bactericidal effect and larger than the smallest dose than can activate the immuneserum.

The antigen has always been derived from agar slope cultures of the bacteria, twenty-four hours old.

The reaction is carried out in ordinary test tubes, where the sera are titrated from 0.1 down to about 0.0000008 cc. by halving each dose. The stated necessary dose of complement is added to each tube, whereupon 1/8000 loop full (2 mm. diameter) of living bacteria + 2 drops of beef broth is added, and the tubes are put into the incubator, where they remain at 37°C. for three hours. Now 2 drops are taken out of each tube and inoculated into fluid agar, which is poured out into plate cultures and incubated for about twenty-four hours. The colonies are now counted. As titer is noted the smallest dose of serum, in which less than 100 colonies have developed.

We have to consider three different manifestations of the bactericidal effect of sera upon the bacteria, namely: (1) The effect of normal, active sera (the total bactericidal effect); (2) The effect of normal inactivated sera, added complement; (3) The effect of normal immunesera, inactivated and added complement.

1. A large number of fresh guinea-pig sera have been examined, some rabbit, and some human sera. With great regularity the sera have shown the same effect on the same groups of bacilli. The group A (the Shiga strain) and the group C (the Danish group III) have been very sensitive to the active serum, which always shows a high degree of bactericidal effect upon these microbes. Group B (the Danish group I and II), however, are less sensitive than A and C. Especially in group C this reaction seems to be of great value. It ought therefore to be used when a microbe, that evidently belongs to this group cannot be otherwise identified.

The result of several tests are put down in table 4.

2. Tested in normal, inactivated rabbit sera, plus the necessary dose of complement (titration of normal amboceptor) Group A and group B showed a fairly high bactericidal titre (down to 0.001 to 0.0002 cc. of serum), while group C was always

TABLE 4

Total bactericidal effect of normal sera from guinea-pigs

GROUPS	STRAINS	NUMBER OF SERA TRIED	BACTERICIDAL EFFECT ON THE STRAINS IN DOSES OF SERA				
			0.1	0.05	0.025	0.0125	0.0063
A	Dys. Shiga	4	+	+	+	+	÷
		2	+	+	÷	÷	÷
B — Danish group I	O36	6	÷	÷	÷	÷	÷
	F11	3	÷	÷	÷	÷	÷
		1	+	+	÷	÷	÷
	F13	2	÷	÷	÷	÷	÷
		1	+	+	÷	÷	÷
	F16	2	÷	÷	÷	÷	÷
		1	+	÷	÷	÷	÷
	F51	1	+	÷	÷	÷	÷
		1	÷	÷	÷	÷	÷
	F52	3	÷	÷	÷	÷	÷
		1	+	÷	÷	÷	÷
	F55	1	÷	÷	÷	÷	÷
	F60	1	÷	÷	÷	÷	÷
	F61	1	÷	÷	÷	÷	÷
	F90	1	+	+	+	÷	÷
B — Danish group II	F29	3	+	+	+	÷	÷
		1	+	+	÷	÷	÷
		2	÷	÷	÷	÷	÷
	F30	1	+	+	+	÷	÷
	F31	1	+	+	+	+	÷
	F	2	+	+	+	÷	÷
		2	+	+	÷	÷	÷
		3	?	÷	÷	÷	÷
	F57	1	÷	÷	÷	÷	÷
	F92	1	÷	÷	÷	÷	÷
C — Group III	F40	3	+	+	+	+	÷
		1	+	+	+	÷	÷
	F41	1	+	+	+	+	+
		2	+	+	+	+	÷
	F71	1	+	+	+	+	÷
	F76	1	+	+	+	+	÷
		1	+	+	+	÷	÷
	F77	1	+	+	+	+	÷
		1	÷	÷	÷	÷	÷
	F79	1	+	+	+	+	÷
		1	+	+	+	÷	÷
	F86	1	+	+	+	+	+
		1	+	+	+	+	÷
	I	1	+	+	÷	÷	÷
	V	1	+	+	+	+	÷
	VI	1	+	+	+	+	÷
	VII	1	÷	÷	÷	÷	÷
	IX	1	+	+	+	÷	÷

quite unaffected by the normal, inactivated serum.[2] Thus it must be assumed that normal rabbit sera contain amboceptor-like substances for group A and B, but no such substances for group C. Consequently a low titre of bactericidal effect of a rabbit serum against the former groups cannot be taken as any sign of specificity, while this must be the case in group C, even if the titre is low.

3. The bactericidal effect of the immunesera upon the various groups of bacilli showed exactly the same relationship between the groups as the agglutination and, the complement absorption tests. The strains isolated in our material fell into the same groups B and C as described before. In group B there was some difference between the strains corresponding to the Danish groups I and II, but this difference was not found important enough to split the group. Group C held the same isolated position as described under the agglunination and complement absorption tests. The bactericidal titres seem to be highest in groups A and B. But allowing for the normal bactericidal sensitiveness of these groups, one will find that the specific bactericidal reaction of group C is quite as high, though the doses of serum required to bring forth the bactericidal effect are larger in this group, where no normal sensitiveness supplements the specific one. As to group A (the Shiga strain), no specific reaction of this group in serum from group B could be found. On the other hand, group B showed a somewhat higher titre in the serum for group A than in normal rabbit sera, while group C was unaffected by this serum.

In sera from dysentery patients the came relationship between the groups was found as in rabbit sera. Especially in group C the reaction was of great value, and often more certain than the agglutination.

II. MORPHOLOGICAL AND CULTURAL CHARACTERS

The dysentery bacilli of the older types (Shiga, Y, Flexner and Strong types) always produce delicate, sharply contoured colonies on the plate cultures, of the same appearance as the col-

[2] Compare the absolute inagglutinability of this group in normal sera.

onies of the typhoid or paratyphoid bacilli. The new group C Danisb III), however, in this respect differs a good deal from the other groups. This group produces rather large colonies, a little denser than those of the other groups and showing a peculiar crenated irregular edge (fig. 1). The center is usually the densest part of the colony, the peripheral parts getting gradually thinner. Blue colonies of this type growing from a dysentery stool render it very likely that we have to deal with bacilli of this group. There may, however, be seen colonies of other types belong-

FIG. 1. COLONIES OF DYSENTERY BACILLUS OF GROUP III FORTY-EIGHT HOURS OLD, THRICE MAGNIFIED

ing to this group. Sometimes we find them very small and dense, at other times large and dense with an edge growing out like a bunch of hair. Both these colonies show irregularities of agglutination, either being inagglutinable or showing spontaneous agglutination in saline solution. If cultivated for some generations on agar, these colonies will as a rule develop colonies of the ordinary type, both as regards agglutination and growth.

The growth of the isolated strains in mannit media always took place with production of acid without gas, thus characteriz-

ing the strains as belonging to the mannit fermenting group of the dysentery bacilli. In litmus milk there was as a rule a slight production of acid, but never coagulation.

The growth in lactose, maltose, sucrose, litmus whey and peptonized broth showed results, that make it necessary to deal with the different media separately.

1. Growth in lactose media

None of the strains showed any production of acid on the first plate culture from the stool after twenty-four hours of growth. A couple of strains belonging to group C, however, showed a peculiar behaviour on continued growth, that made it necessary to investigate more closely the fermentation of lactose by this group of dysentery bacilli. It was found that several of the strains of this group by prolonged growth were capable of producing acid in this sugar, as well in lactose agar (1 per cent) as in lactose broth. In one instance this was found in a first culture from a typical case of dysentery, that showed a clean growth of blue colonies of group C after twenty-four hours. After forty-eight hours of growth, however, the colonies had turned red, having produced acid as if it had been a culture of *Bact. coli*. In no strain belonging to our group B could any such faculty of fermenting the lactose be detected.

The use everywhere of lactose media for differentiating *Bact. coli* from the dysentery bacilli makes it imperative that one be aware of the possibility that bacilli of group C may to a certain extent be capable of fermenting this sugar or soon may learn to split it.

2. Growth in maltose media (see table 6)

It will be remembered that the fermentation of maltose-media had been the chief point of difference between the Y and the Flexner strains of the dysentery bacilli. This fermentation of maltose has been studied by the different authors partly in solid agar), partly in fluid media (beef broth or peptone water), and the results have been compared. Now the authors seem to pay no

no heed to the differences arising from the different media, although such differences certainly exist.

To compare the behavior of the same strains in solid and in fluid media 9 strains belonging to our groups B and C were inoculated into 1 per cent maltose-agar and maltose-broth, to which litmus was added, the media being slightly alkaline. Out of

TABLE 5

Bactericidal action of immune sera from rabbits (the figures signify the smallest doses of serum that showed bactericidal effect after addition of a convenient dose of complement

TESTED WITH			SERUM FROM NORMAL RABBIT	SERUM FROM RABBITS IMMUNIZED AGAINST			
				A	B		C
Groups		Strains		Dys. Shiga	Danish group I O36	Danish group II F31	Group III F41
A		Dys. Shiga	0.0004	0.0000032	0.0002	0.0002	0.0002
B	Danish group I	Danish I strain	0.0008	0.0001	0.0000032	0.0000032	0.0008
		O36			0.000025	0.0000125	
		F52	0.0063	0.0001	0.0000063	0.0000063	0.0032
		F61			0.000025	0.00005	
	Danish group II	F29	0.0016	0.000025	0.00005	0.0000016	0.0016
		F31	0.0016	0.000025	0.000025	0.0000016	0.0008
C	Group III	Danish III strain	+	+	+	+	0.0001
		F40	+				0.0002
		F41	+	+	+	+	0.0002
		F71	+				0.0002

these 9 strains 3, belonging to group C produced acid as well in solid as in fluid medium, although the fluid cultures showed acid production more strongly and quickly than the solid ones. Of the 6 strains belonging to group B, 2 showed themselves as quite vivid producers of acid in the fluid culture, while the solid cultures remained alkaline. The others were non-fermenters both in solid and in fluid media.

This result shows that it is of little value to state whether maltose is fermented or not, when it is omitted to state, whether a solid or a fluid medium was used. It further shows that only when tested in fluid media can a non-maltose fermenting strain really be pronounced to be such.

The use of solid media is not advisable for another reason. The litmus sugar agar, has a great tendency towards being decolorized, probably on account of the absorption of oxygen by the microbe and the following reduction of the litmus. This decolorization begins at the bottom of the tube and ascends slowly to the top, leaving the agar uncoloured or dirty blue or red. In this condition it is often quite impossible to tell, whether the strain in question has produced any acid or not.

Not only the medium, but also the age of the strain has a great influence upon the fermentation of maltose. Already Lenz, Shiga, Krause and other investigators have stated this fact. In our material this influence of age was remarkable. In tests carried out about three years after the isolation of the first strains, several strains that were non-fermenters on isolation had acquired the faculty of vivid production of acid in maltose. Thus these strains had lost their first character of Y strains and had developed into Flexner strains according to the old nomenclature. In the younger strains, isolated from six months to one year previous to these tests, a growing tendency to ferment the maltose could be detected from one test to the next one.

Thus it is obvious that the dysentery bacilli, even if not fermenting maltose in the first culture, may acquire this faculty after some growth on artificial media. On the other hand two strains in this material which did ferment the maltose very vividly immediately after isolation, lost their fermenting power completely soon afterwards. Only a few strains remained non-fermenters all the time.

The increasing tendency to ferment maltose could be nicely demonstrated in the following experiment: Two strains of bacilli (one fermenting, one not) were cultivated in maltose-broth (1 per cent). From this medium the microbes were spread upon maltose-agar-plates.

In both strains the subcultures developed colonies that had fermented the maltose (turned the blue agar into red) and others that had not. These last colonies, that were perfectly typical in all other respects, were now put through new cultures of maltose broth and from these spread upon maltose-plates. Again the plates developed both kinds of colonies, but now the number of fermenting colonies increased, while the non-fermenting ones were reduced in number. After some new cultures the plates at last developed only fermenting colonies. Thus all the bacilli of these strains learned how to split the maltose after growth in a maltose medium.

When the fermenting of maltose is used in the grouping of the dysentery bacilli, the statement "fermenter" or "non fermenter" is made after a very short time of growth (twenty-four, forty-eight, and seventy-two hours). The fermentation, however, is not always finished at this time. Some strains are very slow fermenters, that require a longer space of time to produce acid enough to alter the reaction of the medium. Other strains certainly do produce acid in a short time, but in such a small quantity, that one feels inclined to put the strains among the nonfermenters. These strains consequently are neither strong fermenters, as the Flexner strains ought to be, nor are they non-fermenters like the Y strains, but take a position between these two types. If therefore, a just grouping according to the fermenting of maltose is to be achieved it is necessary to recognize a group of slight fermenters between the other two. But even this grouping can only be correct at the very moment of grouping. When examined sometime later on, namely, one may find that some of the non-fermenters have slid into the group of the slight fermenters, and some of the latter group into that of the strong fermenters. Consequently this mode of grouping is quite illogical.

Also if compared with the grouping based upon the serological characters the grouping according to the fermenting of maltose is illogical. From table 6 it will be seen that strong fermenters are to be found as well in our group B as in group C. Consequently these strains would have to be put into one group, if

TABLE 6

Fermentation of maltose and sucrose (the figures signify days of the experiments)

GROUPS		STRAINS	MALTOSE BROTH REACTION			SUCROSE BROTH REACTION	
			Alkaline	Slightly acid	Acid	Alkaline	Acid
B	Danish group I	O36			1–22	1–4	5–22
		F11		1–12	13–22	1–4	5–22
		F13	18–22	1–1		1–4	5–22
		F16		1–3	4–22	1–4	5–22
		F51	3–22	1–2		1–4	5–22
		F52		1–17	18–22	1–4	5–22
		F55		1–17	18–22	1–4	5–22
		F60	1–5	5–12	13–30	1–4	5–22
		F61	1–5 20–30	6–19		1–4	5–22
		F72		1–10	11–20	1–2	3–20
		F73		1–10	11–20	1–2	3–20
		F75		1	2–30	1–2	3–20
		F78		1	2–30	1	2–30
		F90		1–30		1–3	4–30
		F107		1–15			1–15
	Danish group II	F29	6–7	1–5	8–22	1–22	
		F30	6–7	1–5	8–22	1–22	
		F31	3–22	1–2		1–22	
		F57	3–22	1–2		1–22	
		F92			1–20	1–20	
		F99		1–20		1–20	
		F103			1–20	1	2–30
		F108	1–12			1–12	
		F109	1–12			1–12	
C	Group III	F40		1–2	3–22	1–3	4–22
		F41		1–2	3–22	1–3	4–22
		F71		1–3	4–22	1–2	3–20
		F76		1	2–30	1–2	3–20
		F77		1	2–30	1–5	6–20
		F79					
		F86		1	2–30	1–2	3–20
		F86			1–30	1–4	5–20
		F97			1–20	1–3	4–20

grouped according to the fermentation. It has, however, already been stated that group B differs so much from group C serologically, that it would be quite impossible to join these groups into one.

After this there can hardly be any doubt as to the worthlessness of the fermentation of maltose as a basis of grouping.

2. Growth in sucrose media (see table 6)

According to the classical scheme of the dysentery bacilli only the Strong type should be able to ferment sucrose. This dogma, however, was shaken by Sonne, who found fermentation of sucrose in both his groups I and III. Aronson found strains that fermented sucrose only and others that fermented sucrose and maltose to the same degree.

In our material several sucrose fermenting strains were isolated belonging to both our groups B and C. In the latter group all the strains were able to split this sugar and kept the faculty. Those of group B, however, seemed to have a tendency to lose this faculty, as they grew older. One lost it already after a couple of subcultures on agar, and another fermented sucrose after twenty-four hours of growth immediately after the isolation, but required four days in a test fourteen days later on. It must also be mentioned that an old Strong strain from Germany propagated in this institute at the time has no power of fermenting sucrose and consequently has lost the right to the Strong name.

In five experiments the fermentation of sucrose was studied and it was found, that the strains corresponding to group I of Sonne as a rule did ferment sucrose, those corresponding to Sonne's group II as a rule did not, while all belonging to our group C (Sonne's group III) were certain fermenters.

Thus the fermentation of sucrose is not so constant except in group C, that it can be fully depended upon. Consequently it has but little value in a scheme of grouping.

3. Production of indol

The production of indol has been examined in beef broth and peptone water, and the reaction carried out with the indol reagent of Ehrlich. The tests showed a distinct and constant difference between the groups. While nearly all the strains belonging to our group B did produce indol in a varying degree, not one of the strains of group C ever showed the slightest sign of indol.

As a rule the most vivid producers of acid were the poorest producers of indol and vice versa. This could be nicely demonstrated in experiments with maltose-broth, where strains, that did not ferment the sugar, had produced indol, while the contrary was seen in strains that did ferment the sugar.

4. Growth in litmus whey

The growth in litmus whey has in our material seemed to be of no use in the differentiation between the groups. As a rule the strains have produced acid after twenty-four hours of growth and stayed acid for the rest of the period of observation. On rare occasions there has been an alteration of the acid reaction after a week of growth into an alkaline one. Once only a strain from group C showed a third alteration of reaction. Having been acid the first week, the reaction was alkaline the second, and then again acid the third week. This is of interest, as Sonne finds the two first alterations regularly in all his strains of groups I and II (our group B), while all his strains of group III (our C) has shown the mentioned third alteration. It may be that this difference is to be accounted for by a difference of media, as Sonne has consistently worked with litmus whey from Kahlbaum, while the litmus whey used in our tests has been prepared in this laboratory.

CONCLUSIONS

The material of this investigation consists of 65 strains of dysentery bacilli, all belonging to the mannit fermenting types of the dysentery bacilli.

The old method of grouping according to the fermentation of different sugar media has been abandoned, as it has been found that this fermentation is subject to variations, that make the value of this grouping illusory.

Serologically our material falls into two groups, B and C.

The characters of B are as follows:

1. Production of rather small colonies quite like the colonies of *Bact. typhi.*

2. Production of acid in maltose and sucrose in fluid media. As a rule it is not found in the quite young strains, while the older ones very often show an increasing tendency of fermenting maltose.

3. Production of indol as a rule.

4. Serologically the group shows some relationship to the Shiga type, but none whatever to group C. The strains of group B show some individual variations in serological respects, but not important enough to justify the splitting of the group into more groups.

The characters of group C are the following:

1. Production of rather large colonies of a very typical aspect.

2. Production of acid in maltose and sucrose and occasionally in lactose.

3. No production of indol.

4. Serologically the group shows no relationship to the other groups of dysentery bacilli. The agglutination of this group is not observed in any normal sera or heterogeneous immune sera. The agglutination titres are rather low and the flocculi of the agglutinated emulsion are very fine. The strains of this group are considerably more sensitive to normal active sera from man and animals than the strains belonging to the other group. In immune sera from rabbits the group shows a very specific complement absorption test and bactericidal reaction.

Group B includes representatives of all the old types of the mannit fermenting dysentery bacilli. If then the Shiga type be counted, all the known dysentery bacilli should fall into three well characterized groups of bacilli. Above we have used the capitals A, B and C for these three groups. But as Kruse in Germany

already has employed these letters to signify different types of mannit fermenting dysentery bacilli, we propose to name the three groups mentioned above as group I, II, and III. Doing this, we adopt the grouping of Sonne with the alteration that Sonne's two first groups are united under the figure II, while the figure I is reserved for the bacillus of Shiga. Group III, however, will consistently be the same as group III of Sonne.

The new grouping I, II and III will be in full accordance with the chronology of the dysentery bacilli, the Shiga type being the

GROUPS	SEROLOGICALLY	CULTURALLY				
		Colonies	Mannit	Maltose	Saccharose	Indol
Group I (Bacillus of Shiga)	Specific. Very slight relationship to group II by agglutination, none by other sero-reactions	Rather small. Sharp, regular edge	÷	÷	÷	÷
Group II (Flexner, Y and Strong types)	Slight relationship to group I. Some individual variations of strains	Rather small. Sharp, regular edge	+	+ or ÷	÷ Or rarely +	+ (Seldom ÷)
Group III (Bacillus of Sonne)	Specific No relationship to other groups	Rather large. Uneven, irregular edge	+	+	+ (After some days)	÷

first one isolated, then the strains belonging to the old types Y, Flexner and Strong and at last the new type of Sonne. Further this mode of grouping will simplify the nomenclature by omitting all the names of authors and neglecting the smaller, insignificant variations of the strains that have given rise to all the different old "types." The new grouping will also bring the Shiga type closer up to the other dysentery bacilli, and thereby put an end to such illogical names as pseudodysenterybacilli, paradysenterybacilli, atoxic bacilli, etc. and render it unneces-

sary to use such long names as mannit fermenting or non mannitfermenting types. We propose hence only to talk about three groups of dysentery bacilli, namely:

Group I. The Shiga type (non mannit fermenting, toxic type).

Group II. The Flexner, Strong and Y type (mannit fermenting, atoxic type, pseudodysentery bacilli, Sonne's groups I and II).

Group III. Group III of Sonne (the new member of the mannit fermenting types).

The main characters of these groups are as shown in the tabulated scheme on page 376.

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STUDIES OF THE DIPHTHERIA BACILLUS IN CULTURE[1]

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Received for publication, February 16, 1919

The art of producing toxin from the diphtheria bacillus in culture is apparently very imperfect. While this procedure is one of the most important of the applications of bacteriology in the production of protective serum it has always remained an uncertain one. For every liter of toxin obtained of sufficient strength for immunizing purposes, many liters have been thrown out. Much effort has been expended endeavoring to overcome the uncertainty of the method by those interested commercially in the production of diphtheria antitoxin. In no other field of bacteriological endeavor may it more clearly be seen that the art of bacteriology is in its infancy. In spite of the best efforts to standardize procedure, failures are met with that cannot be explained. We are working with imperfect tools and largely in the dark. More scientific investigation of the essential nature of toxin and the mechanism of its production is necessary before we shall know where the mistakes occur which undoubtedly do occur. In the meantime, it is essential that every new advance in the science of bacteriology which may be applied to this problem, shall be investigated.

One of the most striking contributions to the science and art of bacteriology in recent years has been the demonstration by Clark and others of the effects of hydrogen-ion concentration upon the activities of bacteria in culture. By means of the hydrogen electrode or other suitable means for measuring ionized

[1] Presented before the Society of American Bacteriologists, in parts, December, 1916, December, 1917, and December, 1918.

hydrogen in solution we are able to determine in a much more exact manner than heretofore possible, the true acidity of culture media.

The art of culturing the diphtheria bacillus for toxin production has been built upon studies depending upon the use of litmus as an indicator of reaction. When one considers how inperfect is this manner of measuring reaction one wonders that favorable results were achieved at all. Successes have clearly been won in spite of crude methods.

THE HYDROGEN-ION CONCENTRATION OF THE MEDIUM

A study of the hydrogen-ion concentrations of culture media used for the production of toxin from the diphtheria bacillus was begun in this laboratory in 1916, and certain facts not hitherto mentioned in the literature were brought to light, which facts have, from time to time, been presented orally before the Society of American Bacteriologists. These observations have since been repeatedly confirmed, and it seems worth while to publish a record of them in order that others may have access to this information.

The first medium investigated was a simple meat infusion, enriched with Witte peptone because Witte peptone had always been used in past work. The infusion was prepared as follows:

Veal infusion base

Chopped veal	1 pound
Distilled water	1000 cc.

Soaked overnight; next day heated to coagulate the proteins; juice expressed and made up to 1000 cc.

In our first experiment this veal infusion base was dispensed in Erlenmeyer flasks containing 200 cc. each. To three of these flasks Witte peptone was added to make 0.5, 2 and 4 per cent respectively of the total volume. One-half per cent of sodium chloride was added to each. The three flasks and the control flask (containing no peptone) were then titrated against phenolphthalein in the usual manner, using N/20 sodium hydroxide in boiling solution; and the hydrogen-ion concentration of each

flask was also determined by the hydrogen electrode. The results are given in figure 1.

It will be noted that the addition of Witte peptone to a veal infusion increases the titratable acidity of the medium in direct proportion to the amount of peptone added. At the same time ionization of hydrogen is depressed, resulting in a lowering of the hydrogen-ion concentration and a decrease of actual acidity. This phenomenon is explainable by attributing the lowering

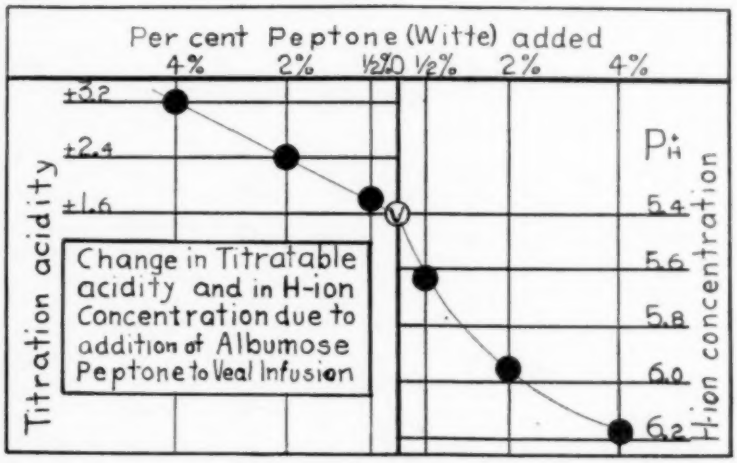

FIG. 1

of the actual hydrogen-ion concentration to the buffer action exerted by proteins in solution and especially by albumoses, in which Witte peptone is rich.

Here is a case, then, in which the method of determining reaction by titration is directly misleading, since the addition of peptone in varying amounts in this case really lowered the H-concentration although the titration value indicated that the acidity had been increasd.

When the flasks as above prepared were "adjusted" with NaOH it was found that the changes in the titratable acidity and in hydrogen-ion concentration did not run parallel. Accordingly the following experiment was set up to indicate what

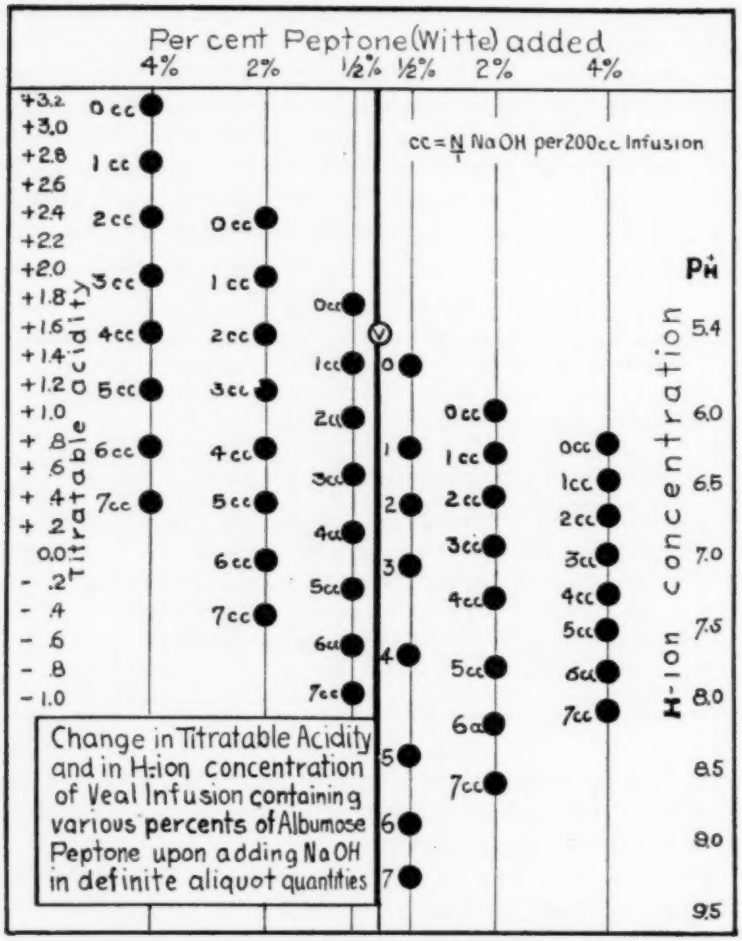

FIG. 2

changes take place in different media upon adjustment of reaction:

Three series of eight flasks each containing 180 cc. of veal infusion base were prepared. The series contained 0.5, 2, and 4 per cent of Witte peptone, respectively. To the members of each

of the series were added 0, 1, 2, 3, 4, 5, 6, and 7 cc. per flask respectively of normal NaOH and the volume made up to 200 cc. in each case with suitable peptone veal infusion. The flasks were then titrated and the H-ion concentrations taken. The results of these adjustments are plotted in figure 2.

It will be noted that the original veal infusion without peptone had a titratable value of + 1.6 and a pH value of 5.4. The acidity in all flasks was lowered by the addition of NaOH as measured both by titration and by the hydrogen electrode. The decrease in acidity as measured by titration was directly proportional to the amount of standard alkali added irrespective of the quantity of peptone in the medium.

The depression of H-ion concentration by the addition of alkali is regulated by the amount of peptone present; the more peptone present, the less the effect of a given aliquot of standard alkali. This is shown in figure 2, where the total change of H-ion concentration in 0.5 per cent peptone broth is indicated as from pH 5.63 to pH 9.20, whereas the addition of the same amount of alkali to 4 per cent peptone broth changed the pH from 6.16 to 8.06 only. Again the effect of buffer action is seen. In those media which contained more peptone, the addition of a given amount of alkali resulted in a smaller change in actual H-ion concentration than in those media containing less peptone and therefore less buffer.

This is another illustration of the fact that the true reaction of culture media is indicated by H-ion concentration measurements and that the values obtained by titration are merely apparent and are often misleading.

In order to show the effect upon true reaction due to sterilization of the media, the following experiment, T-47, is recorded:

Sugar-free veal infusion was used as a base. Witte peptone 2 per cent and 4 per cent was added, and in each case, 0.5 per cent sodium chloride. Two series of flasks were prepared, and the following laboratory record shows the changes which took place upon sterilization:

Table showing changes in reaction during preparation of culture media. Base = sugar-free veal infusion; 100 cc. per flask

FLASK NUMBER	WITTE PEPTONE	N/1 NaOH ADDED	REACTION BEFORE STERILIZING 15 MINUTES AT 15 POUNDS		REACTION AFTER STERILIZING 15 MINUTES AT 15 POUNDS	
			Hot titer	pH	Hot titer	pH
	per cent	*cc.*				
Stock	0	0	+2.7	4.96		
A-0	2	0	+3.4	5.69	3.1	5.69
1	2	1	+2.5	6.28	2.5	6.33
2	2	2	+1.3	6.98	1.7	6.89
3	2	3	+0.6	7.82	1.1	7.55
4	2	4	0.0	8.51	0.3	8.02
5	2	5	*	9.05	†	8.39
B-0	4	0	+4.0	5.97	4.5	5.86
1	4	1	3.5	6.43	3.4	6.43
2	4	2	2.3	7.08	2.8	6.83
3	4	3	1.6	7.56	2.0	7.39
4	4	4	0.5	8.12	1.2	7.75
5	4	5	†	8.63	0.3	8.19

* Distinctly alkaline.
† Faintly alkaline.

In the case of peptone veal infusion, the change in H-ion concentration upon sterilization is in the direction of increasing the acidity. In the more alkaline reactions the change is geatest. In the region of the optimum reaction for diphtheria growth, the change is equivalent to about 0.2 to 0.3 on the pH scale as borne out by experience. Always the change upon sterilization has been in the direction of increased acidity.

The results plotted in this table show that sterilization increases both the titratable and the actual acidity of media, also the fact that a given titratable value does not necessarily correspond to any given pH value, except that at the titratable value of "neutral to phenolphthalein" the pH value will, of course, be very close to the value pH = 8.5. This is necessarily true because unless the dissociation of hydrogen in the medium were of the value pH = 8.5, the medium would not be neutral to the particular indicator used—phenolphthalein. This

relation of titratable value to pH value has been pointed out before, but apparently is not generally recognized, namely that there is only one H-ion concentration at which the pH value and the titratable value of the solution for a given indicator will coincide, i.e.—the point at which the indicator undergoes the greatest dissociation and at which the color change takes place, a point which we commonly call the "neutral point" of that indicator.

TRUE REACTION AND GROWTH

The fact that reaction of culture media has an effect upon the growth and behavior of the diphtheria bacillus is too well known to merit discussion or to require support. It is true that reports of the effect of reaction of culture media have been based upon measurements of reaction made with a very uncertain point of reference, usually litmus. Nevertheless, empirical experiments have been made so many times that eventually we have been able to indicate how reaction as recorded by litmus does affect growth and metabolism under arbitrarily fixed conditions. Endeavors to duplicate any reaction studies made with litmus are subject to error because of the unavoidable differences in buffer content of various media. Nevertheless, the fact remains that it has been demonstrated that reaction does affect growth and metabolism, and therefore we know that reaction must affect toxin production.

In the light of present knowledge, it seems evident that this reaction effect must be due to hydrogen-ion concentration and therefore cannot be estimated by measurements of reaction which depend upon titration. To demonstrate this point beyond doubt, and to determine what are the limits of actual reaction or hydrogen-ion concentration within which favorable growth may be expected, flasks of veal infusion peptone broth, prepared and adjusted in an arbitrary manner as previously described, together with other flasks of similar medium, were together inoculated and incubated.

The flasks were observed daily, and observations of growth recorded. The most rapid development of pellicle took place

on those culture media having an initial reaction in the range pH = 5.6 to pH = 8.8. No attempt was made to sharply define these limits. Later on it was found that the diphtheria bacillus will grow beyond this range, both on the acid side and on the alkaline side after it has a start. The reactions mentioned were those observed to govern approximately the start of vigorous growth.

Previous to inoculation, both the H-ion concentration and the phenolphthalein titratable value of each flask were taken and recorded. Without repeating all of these results, some of the significant values are displayed in the following table:

Table showing the pH and titratable acidity of media which support growth of the diphtheria bacillus. All media are from the same veal infusion base

PEPTONE	HOT PHENOLPHTHALEIN TITRATION	pH	CHARACTER OF GROWTH
per cent			
0.5	+1.0	6.7	Fair
2.0	+1.2	6.9	Fair
4.0	+2.0	6.95	Fair
0.5	+0.2	7.65	Heavy
2.0	+0.4	7.7	Heavy
4.0	+0.8	7.8	Heavy
0.5	−0.2	8.4	Heavy
2.0	−0.1	8.4	Heavy
4.0	0.0	8.4	Heavy

It will be seen that the only place in which there is an agreement between the titratable value and the pH value is at the neutral point to phenolphthalein. At all reactions, the titratable acidities vary according as the buffer content of the medium varies.

It appears then that the reaction of a culture medium, as it affects growth of the diphtheria bacillus, cannot be accurately measured by titration, but that it is truly indicated by determination of the H-ion concentration of the medium.

THE EFFECT OF INITIAL HYDROGEN-ION CONCENTRATION UPON GROWTH

Having demonstrated that H-ion concentration in the medium is one factor which controls growth, it was next in order to determine the optimum reaction in culture media as ordinarily prepared for toxin production.

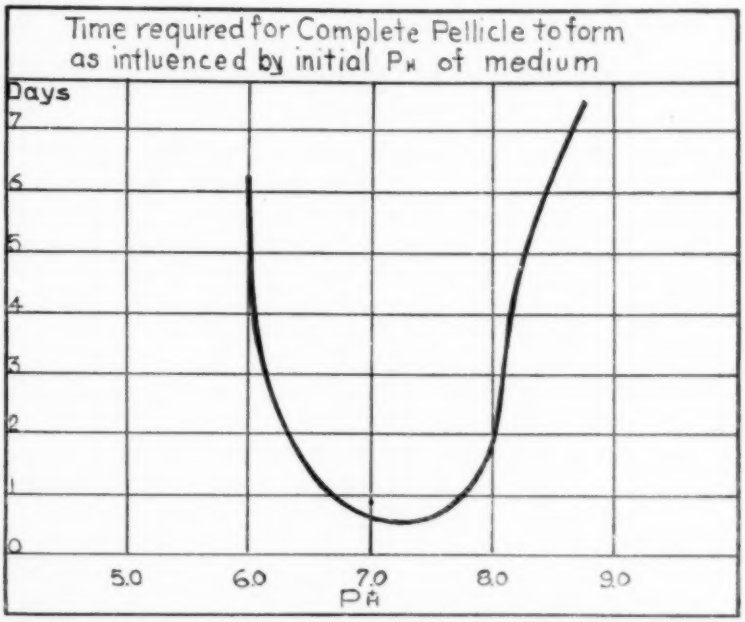

FIG. 3

The medium studied first was prepared as follows; Lean veal, chopped, was infused overnight in the ratio of 1 pound per liter of water, with a vigorous broth culture of *B. coli*, to remove the fermentable carbohydrates. After heating and clarifying, 2 per cent of Witte peptone and 0.5 per cent sodium chloride were added. No glucose was used. The medium was divided into 200 cc. portions in 10 ounce Erlenmeyer flasks in which it made a layer approximately $\frac{5}{8}$ inch deep.

After adjustment to various points over a range from pH 5.0 to pH 9.5 the flasks were sterilized, the final pH taken, and after inoculation, were incubated at 36°C. The time taken to form a complete pellicle over the whole surface was observed, and at the end of seven days, the relative thickness of the pellicle was estimated and recorded.

The results are plotted in figures 3 and 4.

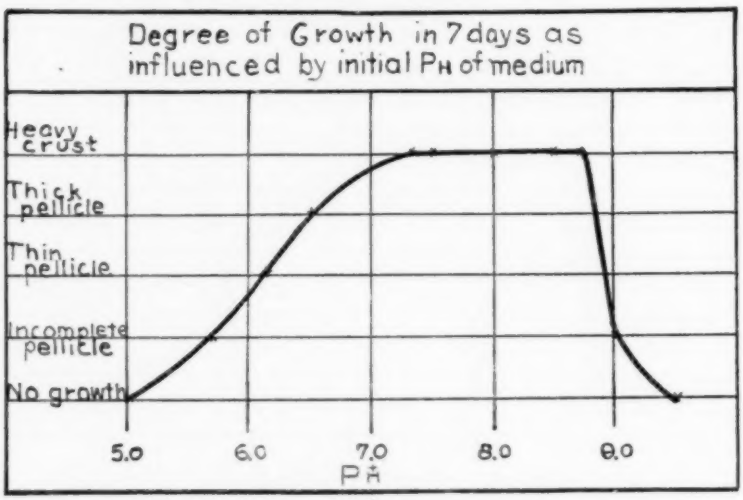

FIG. 4

It is apparent that the initial pH value of the medium affects the speed of growth as shown by the time required to form a continuous layer over the entire surface of the medium. Flasks in the acid end of the series with an initial pH more acid than 6.0 did not form a complete pellicle. At this reaction a thin pellicle formed slowly if at all. Between pH 6.7 and pH 7.7 a firm film would be formed in twenty-four hours, and between 7.0 and 7.5 it would be complete in from twelve to fourteen hours. At pH 8.0 the start of growth was delayed and the pellicle would not be completed until the second day. With the initial pH = 8.8 a week or more would elapse before the surface of the medium was covered.

The appearance of the growth too, differed with the initial H-ion concentration. Growths at the acid end of the series were in general thin and weak, while those at the alkaline end became thick and piled up. This is indicated in figure 4.

At the initial pH = 5.0 there was no growth. At 5.7 the surface growth at the end of a week was in patches and islands which did not cover the surface. At pH 6.0 a thin pellicle would sometimes be spread over the medium in seven days. At pH 6.5 a thin pellicle would be formed of firmer texture, and at 7.3 to 8.7 there would be formed a heavy white crust by the end of the week.

It would appear, therefore, that the optimum initial reaction for growth under the conditions described, lies between pH 7.0 and 7.5; and that the range 7.3 to 7.5 indicates the optimum initial reaction for the most rapid development of the heaviest pellicle.

THE CHANGE IN HYDROGEN-ION CONCENTRATION DURING GROWTH

It is known that the reaction of the culture medium changes during growth, usually becoming first more acid and then more alkaline. A study with the hydrogen electrode of daily reactions, shows considerable regularity and uniformity in this change which takes place, when expressed in terms of hydrogen-ion concentration. The nature of the change is dependent partly on the initial H-ion concentration and partly upon the substances in solution in the medium. This is necessarily to be expected since the reaction change results from growth, and since growth, and metabolism in turn, are profoundly affected by the reaction of the medium and by the nature of food substances available.

When the initial H-ion concentration is favorable for growth, there is first an acid rise in concentration followed by an alkaline reversion which proceeds rather directly to the region around pH = 8.0 and then more slowly to a limiting reaction of lower acidity. If the initial reaction is too acid there will be no growth, or at first a feeble growth accompanied by an acid rise which may proceed to an upper level which will be maintained

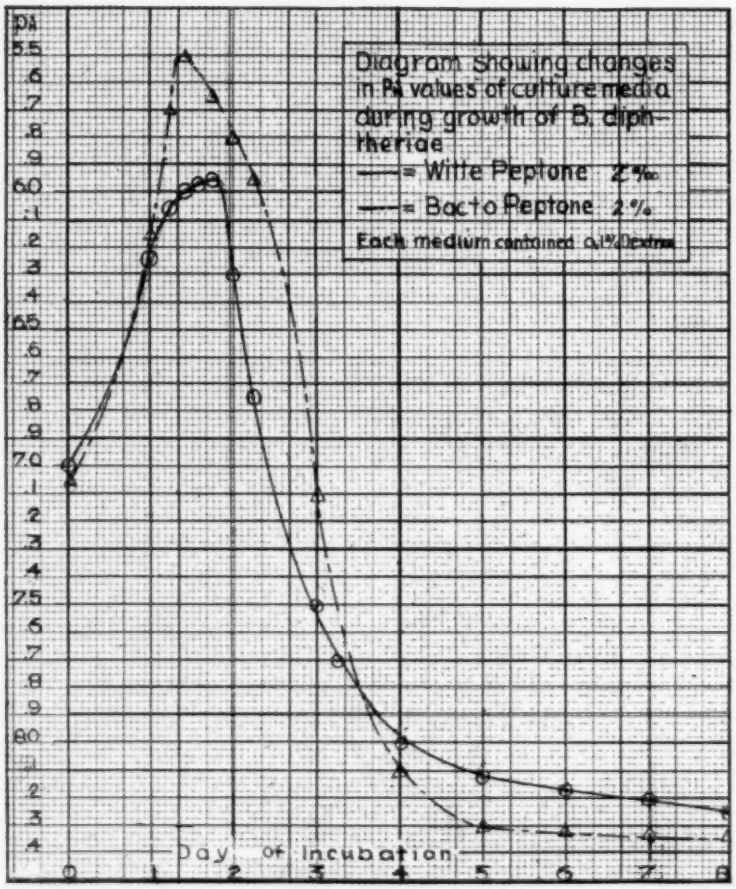
Diagram showing changes in Pᵸ values of culture media during growth of B. diphtheriae
——= Witte Peptone 2%
——= Bacto Peptone 2%
Each medium contained 0.5% Dextrose
Day of Incubation

FIG. 5

until the culture dies. In such cases there is no alkaline reversion, and never in our experience have we obtained toxin from such conditions. Toxin has been gathered only when the typical reaction change from acid to alkali takes place.

Some typical daily reaction changes are plotted and reproduced in figures 5 and 6.

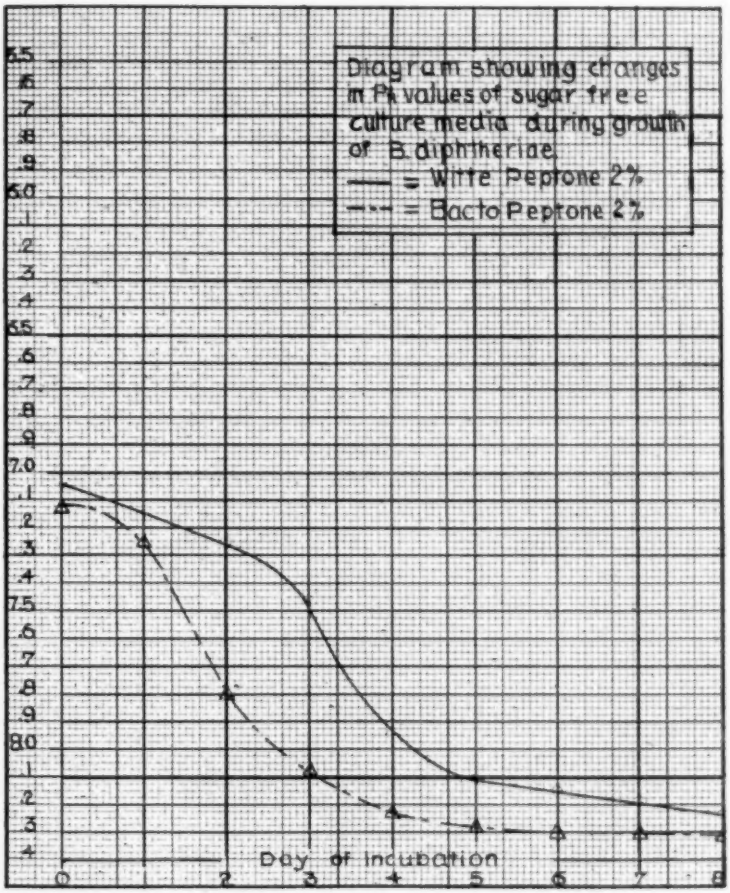

FIG. 6

In figure 5 is shown the plot of pH readings taken at various times over a period of eight days from two flasks, each containing the same veal infusion, each containing 0.1 per cent glucose, one enriched with Witte peptone 2 per cent and the other with Bacto-peptone 2 per cent. This diagram shows, in addition to the shape of the reaction curves formed under such conditions,

the effects of the nature of the peptone used. Witte peptone contains large quantities of albumoses. Bacto-peptone contains none. The acid rise in Witte peptone media is less than the acid rise in Bacto-peptone media under identical conditions. This is to be expected and is explained by the restraining action of the buffer substances in Witte peptone upon the ionization of acid. Although the acid ionization in the case of Bacto-peptone medium is greater than that with Witte peptone, the total change of reaction in Bacto-peptone goes to a more alkaline region than does Witte peptone. This again is attributed to buffer relations.

With different sized flasks and with other cultural conditions, curves of different shapes will be obtained, but in general a typical reaction curve in terms of H-ion concentration will follow the course marked out in diagram 5.

In figure 6 there are plotted daily reaction changes upon sugar free culture media, differing only from the previous flasks in regard to sugar content. The same lot of Witte peptone and the same lot of Bacto-peptone and the same quantities were used, the same incubation temperature was employed and the same culture.

The results obtained are typical of what one may expect under these conditions. So-called sugar-free media are often not free from fermentable substances, and if the media employed are not sugar-free there will always be an indication of this by a slight acid rise previous to the alkaline drop. In the two series which are reported, no acid rise was observed, but it should be remembered that no H-ion determinations were made during the first twenty-four hours of growth, so that a slight acid rise, if it occurred at this time, escaped our attention. The point which this diagram is intended to bring out, however, is that when a sugar-free medium is used and a carbohydrate-free peptone employed, there will not be the usual high production of acid previous to the alkaline reversion; rather the medium tends to become slowly alkaline and then abruptly alkaline and finally it will end up in the same general region of hydrogen-ion concentration reached by those media which contain sugar, this H-ion concentration being the lower limiting concentration for growth.

TOXIN PRODUCTION

With these facts at hand it seemed advisable that attention should next be turned toward an analytical study of reaction conditions during toxin production. The repeatedly confirmed observations of Park and Williams in 1896 concerning the apparent correlation between reaction changes in the substrate and toxin production offered a good starting point. Their work was repeated in part and again confirmed in the main. The record of H-ion reactions thoughout these experiments gave a new set of criteria and a new method of specifying favorable conditions and unfavorable conditions insofar as reaction is concerned. For instance, the hydrogen electrode shows why these investigators found one optimum titratable reaction for media containing 1 per cent Witte peptone and another titratable reaction to be optimum when 4 per cent of peptone was used. The reason is that it is necessary in adjusting media containing different amounts of peptone, to add varying amounts of alkali to compensate for different degrees of buffer action in order to arrive at the one optimum H-ion concentration. Actually the reactions were the same or very similar, irrespective of the way in which they were adjusted, and in spite of the fact that titratable values were different.

A continued study of reaction changes during growth showed that there is a typical shape to the curve formed by plotting periodical readings when toxin is being produced, and that if any set of conditions causes the reaction changes to differ and form an atypical curve, toxin production is apparently affected adversely and may be entirely prevented.

In general, when toxin is produced the reaction changes follow the course indicated in figures 5 and 6, depending upon whether fermentable substances are present in the medium or not.

When for some reason the alkaline drop of hydrogen-ion concentration does not take place, toxin is not found. Toxin has been gathered, however, without any recorded preliminary acid rise. The writer is inclined to agree with the view that the addition of sugar merely speeds up initial growth and lends

momentum to the whole chemical reaction involved, and that if a sufficient amount of easily assimilable food substance is present in the medium to bring about this acceleration of growth, the addition of sugar to the medium is not beneficial.

In figure 7 are shown some atypical curves which were accompanied by failure to demonstrate any potent toxins at any time during the experiment.

That the nature of the "peptone" added has a marked influence on growth and the products of growth has been indicated. The effect of the buffer properties of peptone has already been pointed out. The effect of the kind of cleavage products supplied in the "peptone" content seems to have an even more important bearing on the development of by-products such as toxin. Either because of the form of the molecular structure or because of the nature of the food, certain groups of cleavage products stimulated toxin production. Whether this is to be regarded as explained by the agressin theory or whether these groupings stimulate certain metabolic processes indirectly resulting in toxin formation, or whether they supply the necessary atomic or molecular arrangements which enter into toxin formation, I do not know. At present we have only the observation of apparent correlation between the presence of certain groups of cleavage products and the formation of toxin, with the corollary absence of toxin when these groups are absent.

In determining this point, the line of procedure was first to try the available commercial peptones to see which were useful in developing toxin; next to investigate experimentally prepared peptones of different properties from different materials, and to see which of these would support toxin production.

A series of twenty different peptones, many of them made experimentally in this laboratory, were put on test at one time in small flasks such as were used in the preliminary work. H-ion concentration determinations were made daily and samples withdrawn each twenty-four hours. These samples were too many and too small to admit of filtration through Mandler filters so they were tri-cresolized with 0.5 per cent tri-cresol,

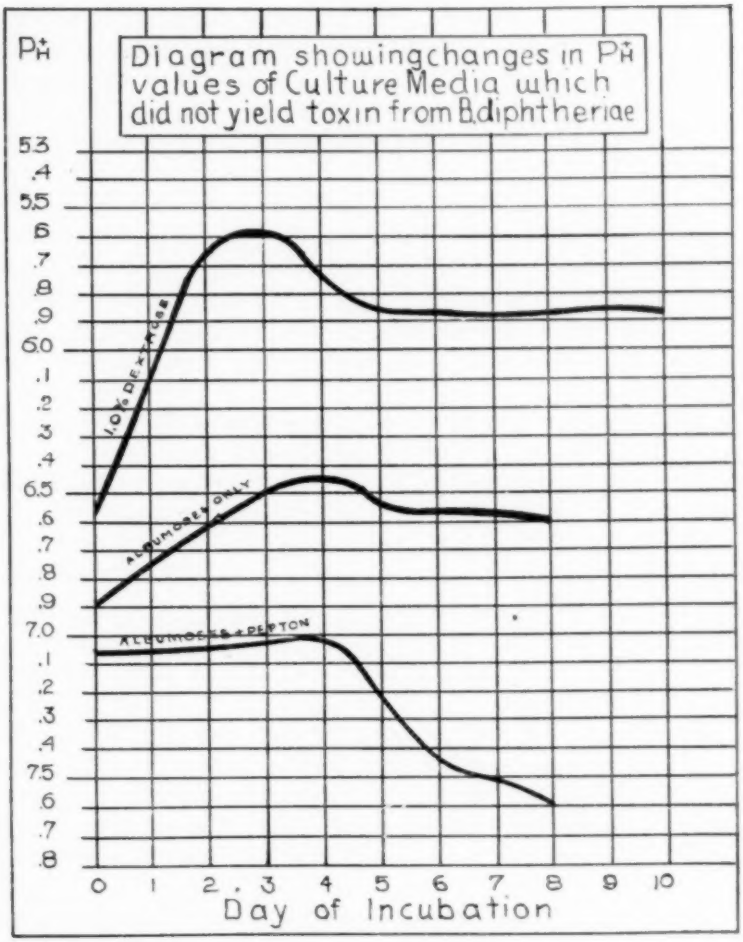

FIG. 7

shaken up, and allowed to stand in the ice-box for several days. Then the supernatant liquor was pipetted off and sealed into ampoules of about 30 cc. capacity. These were stored in the ice-box for a month or so, and were then shipped to one of the large biological manufacturing establishments to be tested on pigs for their minimum lethal dose strengths.

Of the series, Witte peptone developed toxin of the greatest potency, its minimum lethal doses being determined as a little better than 1/300th of a cubic centimeter. Other peptones developed a toxin of minimum lethal dose = 1/200th of a cubic centimeter or 1/100th of a cubic centimeter and many of the samples showed less strength than this. No results were regarded which were less than 1/100th of a cubic centimeter.

When the minimum lethal doses were plotted on the reaction curves, it was found that the most potent toxins occurred in one zone of H-ion concentration outside of which no strong toxins were harvested. Irrespective of the peptone medium from which toxin was obtained, this relation held true in this experiment.

In figure 8 are plotted the reaction curves of six culture media containing different peptones, from five of which toxin was obtained. The numbered circles indicate the points on the curves and the time of incubation at which samples were taken and tested for pH and minimum lethal dose.

In this diagram the peptones used were as follows: A = Witte peptone; B = Blood fibrin—tryptic digestion; C = uncured leather boiled with strong alkali; D = Bacto-peptone; E = Casein peptone—light digestion; F = primary albumoses from Witte peptone (A).

In the case of Witte peptone (curve A), samples taken on the first three days tested less than 0.01 cc. minimum lethal dose. From a theoretical standpoint it would be interesting to know the actual minimum lethal doses of each of these samples. This was a side issue in the investigation and was not carried to completion.

On the fourth day of growth, Witte peptone showed a toxic strength of 0.01 cc. which quantity killed a pig in three days. The pH of the medium at the time this sample was taken was 7.92.

On the fifth day of growth, Witte peptone showed a toxin of minimum lethal dose = 0.003 cc. and pH = 8.1.

On the sixth day the sample was of less strength, 0.005 cc. killing a pig in four days. The pH of the sample was 8.22.

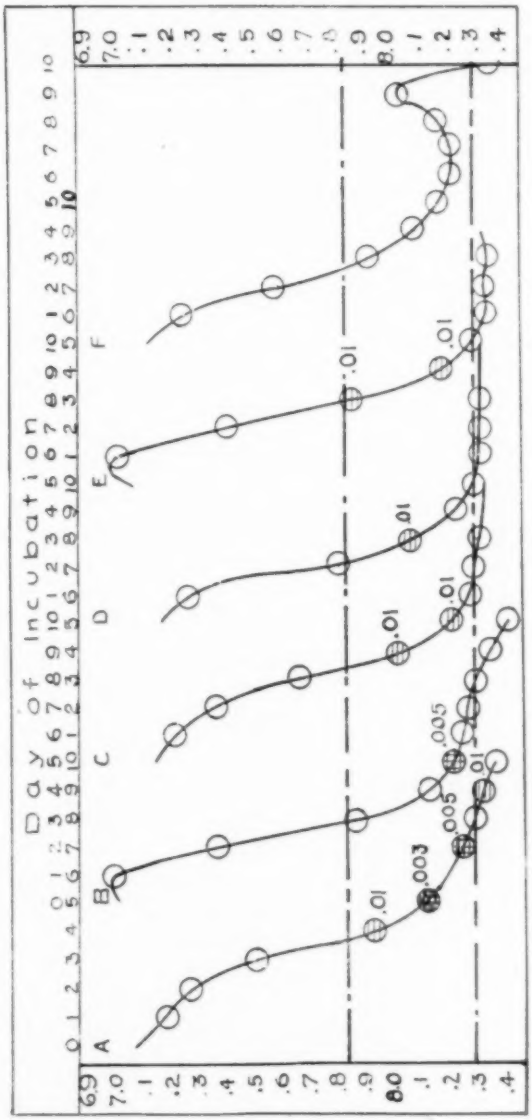

FIG. 8

The seventh day's sample was lost.

On the eight day the sample tested weaker its minimum lethal dose being only 0.01 cc. Its pH was 8.3.

These results with Witte peptone suggest that there is a point at which toxin development is at its maximum, before which and after which the potency will be lost. This point has been noted by previous investigators in terms of time elapsed in incubation. Present attempts to produce toxin call for incubation for a certain number of days before harvesting, still retaining the unit of time. This experiment suggests another possible criterion, namely, final H-ion concentration of the medium rather than age of the culture.

In curve B, again the strongest toxin was harvested on the fifth day of incubation at pH 8.2, the minimum lethal dose being 0.005 cc. Samples on the fourth day at pH 8.22 did not yield potent toxin.

In curve C, minimum lethal doses of 0.01 cc. were obtained on samples gathered on both the fourth and fifth days at pH 8.0 and 8.2 respectively.

In curve D, the sample gathered on the third day at pH 8.05 had a strength of 0.01 cc. Samples on the day previous and on the subsequent day had respectively pH's of 7.8 and 8.2 and neither one tested to a strength as high as 0.01 cc.

In curve E, two toxins of minimum lethal dose strength = 0.01 cc. were harvested on the third and fourth days respectively with H-ion concentrations of 7.85 and 8.1. Samples taken on the second, and on the fifth day from the same flask, did not show toxin of recordable strength.

In curve F, which is the curve obtained by recording the daily reaction changes in a medium enriched only with the albumose fraction of Witte peptone, obtained by half saturated ammonium sulphate precipitation, all samples taken failed to show any recordable toxic strength. Growth seemed rather sluggish and the reaction curve at the end of the run is atypical. It is not impossible that the peak on the ninth day of incubation may be the result of contamination. This was not looked for at the time and later it was not possible to determine the point.

Having plotted these results with the other fourteen of the series on a common scale, it became evident at a glance that all the strongest toxins had occurred at a given range of pH values on the respective curves, and that it would be possible to establish a zone which would include all the strong toxins and outside of which no potent toxin would occur. This zone was arbitrarily fixed by inspection as lying approximately between pH 7.85 and pH 8.25.

In the series of six experiments recorded and plotted in figure 10, it will be observed that no toxins of very great strength were obtained at any time, indicating that some fundamental condition in the experiment was at fault. However, insofar as we could determine, this condition applied to the whole experiment so that results may be tentatively at least taken as comparative. In this experiment, the strongest toxins obtained were in the case of curve A, on the fifth day, in the case of curve B on the fifth day; in the case of curve C on the fourth and fifth days with an indication that possibly a stronger point might have been found between the two; in the case of curve B on the third day with no indication that a stronger toxin could have been expected either before or after this time; in the case of curve E, equally strong toxins on the third and fourth days with nothing to indicate whether a stronger toxin might have been found between the two or after the fourth day sample; and in the case of curve F no strong toxin at all.

Regarding the first five samples which did yield some toxin, it will be seen that in this instance, even though the medium (with the exception of the peptone), the culture, and the incubation temperature are all identical, there is a difference in the time required in these five media to develop the strongest toxin. Regarding the initial H-ion concentration as affecting this, it will be noted that there is some difference in the initial concentration between the five samples examined, but sample C and sample D which both depart from the initial reaction of the other three to the greatest extent, are comparable with each other, and in the case of sample C, the maximum toxin production lies between the fourth and fifth days whereas in sample D,

it distinctly lies at the third day. This offers still further evidence that time of incubation is not the decisive governing factor in determining whether or not toxin will be found, but that reaction, which changes with a more or less constant relation to time in a given medium, is a better criterion.

The curves in figure 8 are governed by conditions present in small flasks where pellicle formation is completed very rapidly. In commercial production, larger flasks are generally employed with a resultant correspondingly increased period of incubation required to reach a given pH value. Under such conditions, the time required for incubation before the correct pH value is reached may be a week or more.

If different peptones are used, different time relations will accompany given pH changes, and if time alone is made the basis of judging when toxin is "ripe," it will be only by chance and in spite of technique that any peptone will give satisfactory results.

In other words, if it be true that the change in pH is affected by peptone, and is correlated to the change in strength of toxin during growth, and that such a correlation does not exist between time and toxin strength, then it is futile to try to duplicate results obtained from one product by applying the same technique in terms of time to another product.

Figure 8 demonstrates this point. If flasks make up with product D were grown for five days, which is the period of time indicated as suitable from experience with product A, whatever the best toxin production of product D might be, it would always be missed, since the maximum production with product D was at three days instead of five.

Another illustration of this is an experiment carried on under production conditions in which due to temperature irregularity a series of flasks which ordinarily should have reached the pH 7.85 in seven or eight days, did not do so until the fourteenth day, when with a pH of 7.83 the minimum lethal dose was 0.005 cc. On the seventh day of this run at which time it would ordinarily be expected from past experience that toxin was ripe, the pH was only 6.8 and 0.01 cc. did not kill a pig. On the ninth

day with a pH = 7.41, 0.01 cc. did not kill. If this experiment had been run on a basis of time, the result would have been to throw away the broth which five days later was of sufficient potency to be useful. It is highly probable that this very thing has occurred many times in the past in the experience of commercial houses where batches of toxin broth have been discarded as having no strength, inasmuch as it has always been the practice of commercial producers as well as of laboratory experimenters, working with diphtheria toxin production to base the experiments upon the basis of time of growth, which, as it appears now, is not a constant factor. In the light of the preliminary work of this investigation which indicated the difference in buffer relations with different media and with different peptones, it will be seen that time and pH changes in the medium do not go hand in hand except for a given medium and for that rare case, a duplication of a given medium in every essential detail.

THE DEVELOPMENT OF A FAVORABLE MEDIUM

With the above observations in mind an attempt has been made to construct a medium which will support quick vigorous growth and at the same time permit a reaction change that will pass slowly through the critical zone, thus giving as much time as possible in which to gather the strongest toxin developed. Such a medium should be well buffered in the regions 7.8 to 8.2, as is 2.0 per cent Witte peptone broth.

The medium containing Witte peptone, as worked out by Park and Williams, will, with a favorable initial reaction, and the ordinary sized flasks, reach the critical zone and remain in it on the sixth, seventh and eighth day of incubation at 36°C. If the initial reaction is far from the favorable pH, as it may well be under existing methods of adjustment of media, time becomes an unreliable indicator of the proper point to gather the product.

It is apparent that many experiments have been discarded, not because toxin was not produced, but because it was not gathered when ripe before "deterioration", or because it was gathered before it had reached its greatest activity.

Broth may be prepared with true peptone and inorganic buffer which will follow in reaction change the favorable path, but we have never gathered strong toxin at any time from such media. Such media unbuffered pass rapidly through the critical harvesting zone, and if toxin is present at all it is there for a short time only, a matter of hours instead of days. It will be noted also that a medium enriched with Witte primary albumose alone, while it is highly buffered and stays in the critical zone for a long time, has not ever in our experience, yielded a potent toxin.

It would seem therefore, that not only must reaction changes be typical and not only must there be a suitable buffer substance in the medium, but there must also be some particular kind or grouping of food substance in order that toxin shall be developed.

An analytical study of Witte peptone and other peptone by an alcohol fractionation method, the technique of which will be published elsewhere, showed the presence of certain groupings of cleavage products in those peptones which gave good results, and the absence of these groupings in peptones giving poor results in toxin production.

Witte peptone has been separated arbitrarily into the following fractions: (1) Water insoluble; (2) soluble in water but insoluble in 50 per cent alcohol; (3) Soluble in 50 per cent alcohol but insoluble in 80 per cent alcohol; (4) soluble in 80 per cent alcohol.

These fractions have been tried separately and in combination to see if one or if any combination of them is of greater value in developing toxin than the whole group. The third fraction gives the most encouraging results, that portion which is soluble in 50 per cent alcohol and insoluble in 80 per cent alcohol. American peptones, some of them designedly so, are low in this fraction and rich in the 80 per cent soluble fraction, which is as it should be for purposes of growth stimulation.

An experimental product made from peptic digestion of meat and processed to eliminate the useless fraction of primary albumoses insoluble in 50 per cent alcohol, but retaining some of the group of polypeptides insoluble in 80 per cent alcohol, gives encouraging results.

A comparative fractionation of this product and Witte peptone made by the same arbitrary alcohol method referred to above, is given herewith:

	INSOLUBLE IN WATER	SOLUBLE IN WATER INSOLUBLE IN 50 PER CENT ALCOHOL	SOLUBLE IN 50 PER CENT ALCOHOL; INSOLUBLE IN 80 PER CENT ALCOHOL	SOLUBLE IN 80 PER CENT ALCOHOL
	per cent	*per cent*	*per cent*	*per cent*
Witte	7.29	26.16	50.14	15.86
Polypeptides	0.90	1.25	55.35	41.22

Growth takes place in a medium containing 2 per cent of this product which we shall call for convenience polypeptides, in a very satisfactory manner.

The reaction curve coincides with that of Witte peptone in 2.0 per cent medium over a period of seven days.

Toxin has been produced as often with this medium as with Witte medium, both in the laboratory over a period of twenty-three months, and under working conditions of commercial production in one of the large biological houses, and once with rather inadequate equipment in a government laboratory to meet an emergency during the war.

The most interesting test is that which extended over a period of one month, and was conducted with standard equipment and incubator space and facilities found at one of the large commercial biological laboratories. During this time, experiments were put on daily for approximately two weeks with the following results: Out of 12 starts with polypeptide medium there resulted minimum lethal doses as follows: One of 0.0025 cc., three of 0.005 cc., three of 0.01 cc., four less than 0.01 or doubtful. At the same time nine Witte controls were run which resulted in minimum lethal doses as follows: One 0.0025 cc., two 0.005 cc., two 0.01 cc., four less than 0.01 or doubtful. This is of course too small a number of experiments upon which to base any statistical analysis, but it will be noted that the percentage of failures to obtain toxin of usable strength in the case of the polypeptide medium is $33\frac{1}{3}$, whereas in a corresponding series

of Witte controls, the percentage of failure is 44. Such manipulation of small groups of figures, however, does not meet with our approval and we are content to draw the conclusion from this series and from other series that in the majority of cases the polypeptide medium is as useful in building potent diphtheria toxin as is Witte peptone.

The buffer content of the medium containing polypeptides is such that the reaction curve is reasonably flat during its passage through the critical zone of reaction. This is indicated by a record which follows:

Experiment 1918—O. Polypeptides medium, initial pH 7.5, temperature 36°C.

HARVESTED ON	MINIMUM LETHAL DOSE	REMARKS
Sixth day	0.005+	Pig died on sixth day
Seventh day	0.005−	Pig died in less than four days
Eighth day	0.005+	Pig died on sixth day
Ninth day	0.01−	
Tenth day	0.01−	
Eleventh day	Lost	
Twelfth day	Less than 0.01	

Some toxin is obtainable under the conditions of this experiment on either the sixth, seventh or eighth day, but it is true that on the seventh day it is stronger than on the day previous or on the subsequent day.

The toxin so prepared has lasting qualities as shown by the following laboratory run:

Experiment 1918—Q. Toxin stored eighty days after gathering before testing

DAY GATHERED	WITTE	POLYPEPTIDES
Sixth day of incubation	0.005−	0.0020
Seventh day of incubation	0.0033−	0.0025
Eighth day of incubation	Less than 0.005	0.0025

The technique used in obtaining these results is as follows: Lean veal is chopped and infused in tap water in the ratio of 1 pound per liter. The mixture of meat and water is made at the

latter end of the afternoon, and is warmed to about 40°C., in a large, open, jacketed kettle. To every 20 gallons of meat-mixture is added about 200 cc. of a broth culture of *B. coli*. In the morning the fermentable substances will have been largely removed if the mixture has not cooled down too much overnight.

Heat is then applied to coagulate the proteins and the infusion strained through several layers of cheesecloth and dispensed into 2-liter Erlenmeyer flasks which are plugged and sterilized in the autoclave for one-half hour at 20 pounds pressure. When it is desired to make up medium from this base, a flask is opened and if a precipitate has been formed in it through sterilization to such a degree as to be undesirable, it is first filtered through paper. Otherwise the desired quantity of veal infusion base is withdrawn and to it are added 0.5 per cent sodium chloride, 2.0 per cent polypeptides and sufficient sodium hydroxide to bring the reaction to pH = 7.8. This medium is then heated (in the autoclave for convenience) for about fifteen minutes at approximately 15 pounds. The purpose of this heating is to coagulate such material as will be precipitated by the change in reaction, and by heat. The precipitated medium is then passed through bibulous filter paper or thick cloth and dispensed in $\frac{1}{2}$ gallon rounded square amber glass bottles, 400 cc. per bottle. These are plugged with cotton, paper or cloth is tied over the necks, and they are sterilized in steam in the usual manner. When it is desired to inoculate the bottles, they are laid on their sides with the neck slightly raised, the covering is removed from the neck, the stopper withdrawn and the Bunsen burner played over the mouth of the flask for a second or two. It is then very simple to transfer a loopful of surface culture by means of a long-handled needle from the broth stock tube to the surface of the liquid in the bottle. We find that this method of floating on the inoculum is superior to pouring in a quantity of broth culture. Bottles are then stoppered and placed on their side in the incubator which is held at a temperature of 36°C.

By using this type of bottle instead of the round flask it is possible to stack bottles as high as incubator space will admit,

one on top of the other and still have plenty of air in each bottle over the surface of the medium to take care of the growth of the diphtheria organisms.

A film should spread over the surface of the liquid in twenty-four hours. Toxin at this temperature will be ripe in about seven days, but some toxin can be gathered on the sixth or the eighth day.

The procedure as outlined will give a medium which has a H-ion concentration of about pH = 7.5 after final sterilization.

CONCLUSIONS

1. The growth of the diphtheria bacillus in culture is influenced by the H-ion concentration of the medium. It is important, therefore, in adjusting culture media that the adjustment be made in terms of H-ion concentration.

2. It has been shown that the diphtheria bacillus will develop on peptone broth of an initial reaction between pH 5.7 and 8.7, and that when growth is started it may pass either of these limits.

3. It has been shown that for one strain at least, any initial reaction between 7.0 and 7.5 will give equally rapid development of a pellicle and that these two limits indicate approximately the range for optimum reaction as regards speed of development of pellicle.

4. It has been shown that at any initial reaction between pH 7.3 and pH 8.7 on ordinary media there will be formed in the course of about seven days, a pellicle of apparently equal weight after a more or less delayed start. It seems, therefore, that for the most rapid development of the heaviest pellicle, an initial reaction of pH 7.3 to 7.5 is most favorable.

5. The normal change of H-ion concentration during development of growth consists of a primary production of acid followed by a secondary alkali production which progresses until the pH 8.0 is reached, and then more slowly to the limiting concentration.

6. It has been observed that the harvesting of strong toxins has taken place within a definite H-ion concentration range

bounded approximately by pH 7.8 and pH 8.25. Witte peptone formerly used by bacteriologists is heavily buffered in this region. American peptones are not. This is suggested as one reason why the attempts to utilize American peptone with a technique based upon the past experience with Witte peptone have not been successful.

7. It has been shown that reaction conditions alone cannot govern toxin production. Besides favorable reaction conditions it is necessary also that suitable food substance be present in the media.

8. A peptic digest of meat having a content of polypeptides insoluble in 80 per cent alcohol but soluble in 50 per cent similar to Witte peptone has given favorable results over a period of two years. In sugar-free veal infusion containing 2 per cent of this product and 0.5 per cent sodium chloride with a strong strain of P.W. No. 8, it is possible to obtain consistently usable toxins.

9. An initial H-ion concentration after final sterilization of pH = 7.5 is indicated as favorable.

10. For every set of conditions, including the size and shape of containers the nature and the activity of the strain used, the incubation temperature, and other factors, it is necessary to work out by experience the time necessary to incubate cultures in order to obtain the most potent toxin. This may be done by means of following the daily H-ion concentration changes in a series of experiments under similar conditions. It will be found that the media, if prepared in the same way from the same materials, and the same conditions of culture applied, will reach a critical range of H-ion concentration between 7.8 and 8.25 approximately at a given time, and that once the time factor has been determined for the reaching of this critical zone, time may be used as a criterion for harvesting toxin from media made up in a similar manner thereafter.

THE GROWTH OF CERTAIN BACTERIA IN MEDIA OF DIFFERENT HYDROGEN ION CONCENTRATIONS[1]

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Received for publication, March 26, 1919

The literature of bacteriology is replete with statements that the "reaction" of a culture medium is important, but important for what particular phases of growth or for what specific processes of metabolism is not yet known in much detail. Recent investigations have made clear certain of the more general aspects of the subject and have demonstrated the value of hydrogen ion determinations; but circumspection must be exercised, and much additional experimental data will be required before the mechanism of the phenomena now known can be explained. The influence of the hydrogen ion on cellular multiplication is probably far from being one of those simple matters which we like to call fundamental; yet it is obvious that we must seek detailed information concerning this phase before studying intensively the metabolism of a culture as a whole over long or short periods and at various pH reactions.

Much of the work contributory to the subject has been reviewed in previous papers from this laboratory (J. Bacteriol., **2**), and in subsequent papers by others. In many of these contributions the authors have dealt chiefly with the lethal or limiting pH, but in others such as those of Shohl and Janney (1917) and of Dernby and Avery (1918) we find data upon rates of growth within a range of pH favorable to growth. The reports of Bunker (1916–1917) on studies with the diphtheria

[1] Published by permission of the Secretary of Agriculture.

A report of this work was read at the Washington meeting of the Society of American Bacteriologists, December, 1917.

bacillus, of Cole and Lloyd (1917) on the gonococcus, of Cullen and Chesney (1918) on pneumococci, of Fred and Loomis (1917) on soil bacteria and of Gillespie (1918) on an organism (causing "potato-scab") whose response to pH is of considerable importance to potato growers, as well as several other recent investigations have all served to outline the field for a survey, the extension of which is greatly needed.

In the present investigation we have attempted to narrow the problem with the hope of obtaining more quantitative results. We set out to determine the influence of pH upon rates of growth during that period in the life history of a bacterial culture commonly known as the period of logarithmic increase. The investigation assumed the aspect of a preliminary survey until it became evident that some uncontrolled factor was shortening the period of logarithmic growth to a disconcerting degree. No attempt was made to remedy this by empirical readjustment of conditions. We continued with the materials and the methods with which we had started because the results seemed to be suggestive and because it seemed desirable to have at least a preliminary survey before attempting a special and systematic search for the unknown factor or factors involved.

The organisms studied were: *B. coli*, laboratory culture 22 fg; *B. aerogenes*, laboratory culture 22aax; *B. dysenteriae* (Flexner), American Museum No. 110; *B. dysenteriae* (Shiga) orginally from Kruse, furnished by the Rockefeller Institute; *B. proteus*, furnished by Miss Bengston, United States Hygienic Laboratory; *B. alcaligenes*, American Museum and *B. bulgaricus*, laboratory culture 39a.

The organisms were cultivated in a peptone phosphate broth for multiplication studies and upon nutrient agar for counts. The broth consisted of 1 per cent "Difco" peptone and 0.5 per cent K_2HPO_4 to which was added HCl or NaOH to obtain the desired pH. The pH values were determined colorimetrically with the indicators and the procedure described by Clark and Lubs (1917a) and with occasional electrometric control (Clark, 1915a). The nutrient agar was adjusted to pH 7.1. The inocula consisted of aliquot volumes of a culture incubated

eighteen hours at 37°C. The medium for this culture was of the same composition as that inoculated, and was adjusted initially to pH 7.1. The multiplication studies were made in 200 cc. portions of the medium warmed to 37°C. before inoculation and held at this temperature during the experiment. Plates were held seventy-two hours at 37°C. before being counted. Frequent tests of pH during an experiment showed that the change in reaction was insignificant during the short period considered in the calculation of rates of logarithmic increase. When it is necessary to state the results of counts taken over periods as long as five to ten hours we shall use the pH value found in each instance at the time a count was made.

By plotting against time the logarithms of the counts it is possible to obtain by inspection of the curve an estimate of the period within which the increase in viable cells approaches the logarithmic law. The counts found during the period so estimated were introduced into the equation: $b = a \times 2^n$. Here b represents the number of organisms at the end of a period T, a the number at the beginning of the period, and n the number of generations during this period. When the length of the period T is expressed in hours, $\frac{n}{T}$ gives the number of generations per hour. These equations are approximate but quite adequate when we consider the large experimental error involved in counting the plates.

It frequently happened, especially at the more extreme pH values, that no three counts fell on a curve which indicated true logarithmic increase. In such cases we have considered the highest average rate computed from three or more successive counts to indicate the magnitude of any logarithmic increase which may have occurred. As an example of the series of values found there are shown in figure 1 the generations per hour plotted as ordinates and computed from counts of *B. coli* cultures held at the pH indicated by the abscissae. The negative rate shown at pH 4.6 means that a logarithmic decline in numbers was observed for a short period in this case.

Not all the experiments furnished data giving such a smooth curve as that shown in figure 1, but in almost every case the same type of curve was obtained with the other organisms. In each there was very plainly evident a broad zone within which relatively high and more or less uniform rates of reproduction were found for short periods. It is also very clear that at the borders of such a zone a comparatively slight shift in pH produces a profound effect.

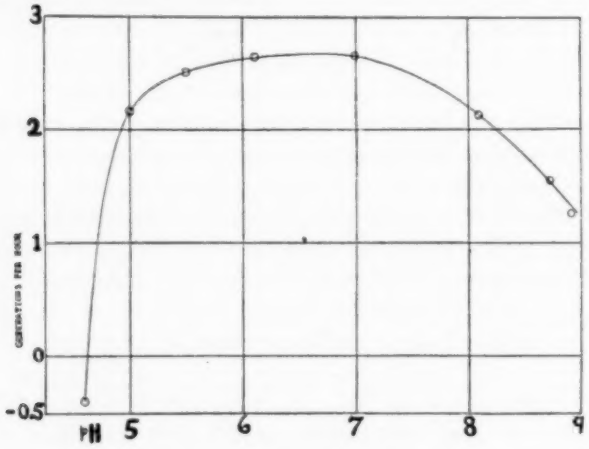

FIG. 1. RATES OF GROWTH OF B. COLI

Average generations per hour plotted as ordinates against pH as abscissae

There is some evidence, too incomplete to dwell upon, that the slope of the curve for *B. alcaligenes* is not abrupt on the acid side as it is for the other organisms studied, and that we have in this case a type of curve which is in this respect distinct. Since there is so plainly evident a broad zone of pH within which relatively high maximal rates of growth were observed for short periods, we may conveniently summarize all the data on this point in table 1.

The chief effect of reaction in so far as we have to do only with the period of maximal rate of increase seems to be confined to the limiting extremes. This is indicated in figure 1, and again in

TABLE 1

Generations per hour for period of logarithmic increase within pH zones indicated

ORGANISM*	pH RANGE	AVERAGE GENERATIONS PER HOUR
B. coli	5.0–8.1	2.5
B. aerogenes	4.7–8.4	2.7
B. dysenteriae (Flexner)	5.6–8.3	1.6
B. dysenteriae (Shiga)	5.5–8.5	1.1
B. proteus	6.5–8.8	1.5
B. alcaligenes	6.9–9.7	0.9

* *B. bulgaricus* is not included in this table because it was studied in a medium of different composition, and the results may not be comparable with those of the other organisms.

table 2. In table 2 are shown first the pH at which the numbers of viable organisms declined,—the decline being expressed as generations per hour with a negative sign. Next are shown the maximal rates of increase at the highest acidity where definite increases were observed. Similarly, the conduct of the organisms in alkaline media just within, and in two cases without the zone where growth was observed are shown in the last columns.

TABLE 2

Rates of increase at pH values indicated

	ON ACID SIDE				ON ALKALINE SIDE			
	pH	Rate*	pH	Rate	pH	Rate	pH	Rate
B. coli	4.6	−0.4	5.0	2.2	8.9	1.3		
B. aerogenes	4.4	−1.0	4.7	2.6	9.0	0.3		
B. dysenteriae (Flexner)			4.8	0.8	8.7	0.9	9.1	−5.6
B. dysenteriae (Shiga)	4.7	−0.6	5.3	0.9	8.8	0.3		
B. proteus	5.4	−1.7	5.6	0.9	8.9	0.2	9.0	−0.1
B. alcaligenes	5.5	−0.3	5.7	0.2	9.7	0.8		

* In generations per hour.

There are some points of special interest to be noted in the fact that, while the organisms can multiply rapidly for short periods in media having a considerable range of pH, a slight change at the border of this zone determines a definite decline in numbers. This illustrates a theoretical principle discussed briefly by Clark and Lubs (1917a) who pointed out that substances whose dis-

sociation is influenced by the pH of a solution would be affected but little within wide zones of pH, and profoundly affected by slight changes at a border or borders of such a zone. In the case now under consideration, the change in the conduct of the cells is more abrupt than might be expected and is, therefore, of special theoretical interest.

Since the change is so sharp it might be supposed that we have in the study of reproduction a method capable of showing a definite limiting pH—a method which is applicable where others are not. For instance, it is specially interesting to note that in our present series of experiments the critical pH for *B. aerogenes* occurs at about 4.5. This organism decomposes organic acids so readily (Ayers and Rupp, 1918) that it is difficult except under accidental or skillfully controlled conditions to force the culture by sugar fermentation to so high an acidity as pH 4.5. It was this fact that Clark and Lubs (1917) took advantage of in designing the so-called methyl-red test. We have obtained values as acid as pH 4.9 by the fermentation method under suitably controlled conditions, but for the reason just stated have never felt as certain of their significance as now when seeking the limits for *B. aerogenes* by the present method. The reproduction studies indicate that *B. aerogenes* and *B. coli* are influenced about alike in their reproduction by the same pH.

On the other hand, the value so obtained can not be considered as a well-defined constant. The fermentative limit was discussed by Clark and Lubs (1917b) who prefer to consider the values experimentally found in particular cases as indicating a limiting *zone*. That such a limiting zone in the case of reproduction may become wide is shown by some experiments in the present series where adjustments of pH were made with acetic acid in place of hydrochloric acid. Distinct differences were observed. For instance at pH 5.0 when obtained with HCl, *B. coli* grew well for a time; but at pH 5.45, when obtained with acetic acid, the same organism barely held its own and finally declined in numbers. The same difference in conduct was observed with other organisms. We did not at that time pursue this subject further in order to determine the conduct of *B. coli* in acetic acid

media of slightly higher pH, but some months later Dr. Sherman kindly repeated the experiment with this end in view. He found in media adjusted with acetic acid that at pH 5.5 there was a slight decline in numbers extending over the first sixteen hours of observation—thus confirming our earlier work. No cloud indicating growth ever developed in this medium. At pH 5.7 he found an almost stationary value for the number of viable cells counted during the first sixteen hours, but at the end of twenty-four hours he found a large increase. At pH 6.0 the same thing occurred except that the increase began at the tenth hour; and at pH 6.2 the increase began between the second and fourth hours. In this case, then, the narrow pH zone 5.5–5.7 may be considered critical,while in the former case where the medium was adjusted with HCl it was 4.6–5.0.

We come now to a brief consideration of the "unknown" factor to which we have already alluded. The question naturally arises: Is it truly an unknown factor, or merely one which we should have recognized and controlled? We are not unmindful of the considerable body of literature bearing upon inhibition of bacterial growth, but we know of no points which are in our opinion sufficiently well established to permit of application to this admittedly brief and preliminary survey. Mature deliberation has led us to the conclusion that it is preferable to consider that we have yet to discover the main cause of the sudden inhibition of growth. Rather than repeat our experiments by a hit or miss method of improvement it seems wiser to let the present results stand for what they are worth until such a time as we can recast the procedure intelligently.

In common with other observers we found the period of logarithmic increase to be of short duration. Evidence of inhibition often appears within four hours after inoculation. Can this be due to an accumulation of by-products of metabolism? To show how difficult the solution of this problem may become by an unsuitable choice of conditions, we shall first describe an experiment with a *B. coli* culture grown in 1 per cent Witte peptone, 0.5 per cent K_2HPO_4 and 1 per cent glucose, and in which pH determinations and counts of viable bacteria were

made simultaneously. The results are plotted in figure 2, where the abscissae are hours, and the ordinates logarithms of the numbers of bacteria (scale at left), and pH values (scale at right). The curve for the sugar-free medium is included for comparison and will be considered presently.

Between the fifth and tenth hours the increase in the number of viable bacteria followed the logarithmic law, but between the tenth and the fourteenth hours inhibition of growth became evident. In this period the pH of the medium approached that

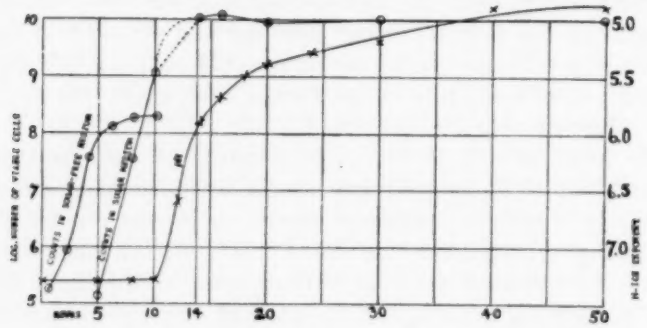

FIG. 2. COMPARISON OF THE GROWTH CURVE OF B. COLI IN PLAIN AND GLUCOSE MEDIA; AND THE ACID DEVELOPMENT IN THE GLUCOSE MEDIUM EXPRESSED AS pH

The abscissae give the time in hours. The ordinates: at left, logarithm of number of viable cells; at right, pH (hydrogen ion "exponent")

region where we have found acetic acid to inhibit growth. Was the check in growth in this instance due to the pH or perhaps to acetic acid which is a product of the sugar fermentation of *B. coli*? The question might have received a clearer answer had frequent and accurate counts been made between the tenth and the fourteenth hours; but the margin of safety in drawing conclusions would have been narrow.

For purposes of comparison there are plotted in figure 2 the logarithms of the numbers of viable *B. coli* in a sugar-free medium whose pH remained practically constant. It is evident from inspection of this curve that a severe check in growth took place early and that it was due to some factor other than pH.

We have then this peculiar situation. As is indicated by the flattening of the pH curve as well as by other facts, the *fermentative activity* in the glucose medium is checked when *B. coli* brings the culture to a zone of pH close to that at which HCl checks growth; but the *growth* of *B. coli* is checked when as it happens in this case, the pH approaches that at which acetic acid, a product of its sugar fermentation, can be made to check growth.

There is considerable evidence, some of which has been presented in previous papers from this laboratory, which points to the conclusion that under ordinary cultural conditions it is the pH which exerts the greatest influence upon acid and gas production by *B. coli*, and that other inhibitory conditions are of secondary importance. It is possible that these other inhibitory conditions may have a strong effect upon the processes of reproduction, but may not make themselves felt to any great extent upon the fermentative activities. When we turn to the experiment with the sugar-free medium in which the pH was held practically constant and favorable to growth, we find growth seriously affected after the fourth hour. Yet the number of viable cells at this moment was but 5 per cent of that in the sugar medium when the growth curve of the latter fell from the logarithmic rate. It is to be strongly emphasized that a comparison of the *effect* of the products in the two cases is unfair, but the alternative assumption that the much smaller number of organisms in the one case could have produced a more active *quantity* of by-products in a shorter time is to be doubted.

It may be suggestive to note that, while the rates of logarithmic increase taken early in the course of the growth curve furnish no distinct optimal pH, those taken over longer periods do. Cole and Lloyd found that in simple media gonococcus required a narrow zone of pH, while in "hormone" media the pH zone within which growth was good was comparatively broad. It is possible that in the simple medium there were rapidly produced those conditions which appear only later in the more complex media.

Chesney (1916) and many others have already dealt with the inhibition of growth, but we feel that much remains to be clarified. We shall not discuss it further, having mentioned it merely

to show that we recognize a difficulty which seriously hampers the clear analysis of our data. However, it seems clear that the influence of this unknown factor will tend to obscure the influence of the hydrogen ion concentration, and that the differences observed indicate at least submaximal effects of pH.

The only variation between the media in the main series was, so far as we know, in the quantity of HCl or NaOH added, but there are two other factors which may have some bearing on the results, and which were not investigated. In the first place, although our inocula contained numbers of viable cells of the same order of magnitude, these numbers were large. Slator (1913) has shown that in the cultivation of yeast cells it is better to use small numbers if logarithmic increases are to be observed. In the second place, attention should be called to the fact that the organisms employed do not grow well on sugar-free media if the air is removed by evacuation. Our experiments were conducted in 200 cc. portions of the medium, and although exposed to the air and shaken frequently before the removal of the samples, there may have been a gradual and irregular transition from aerobic to partially anaerobic conditions.

There still remains for consideration the influence of reaction upon periods other than that of logarithmic increase, as well as upon the whole course of the growth curve.

We noted with *B. coli*, *B. aerogenes*, *B. dysenteriae* (Shiga), *B. proteus* and *B. bulgaricus* that the time of maximal rate of growth was delayed until a late hour in the more alkaline media. With *B. coli* the periods of maximal rate of increase occurred as follows:

pH	PERIOD
	hours
5.0	3–5
5.5	3–5
6.1	2–4
7.0	2–4
8.1	2–4
8.7	8–10
8.9	10–12

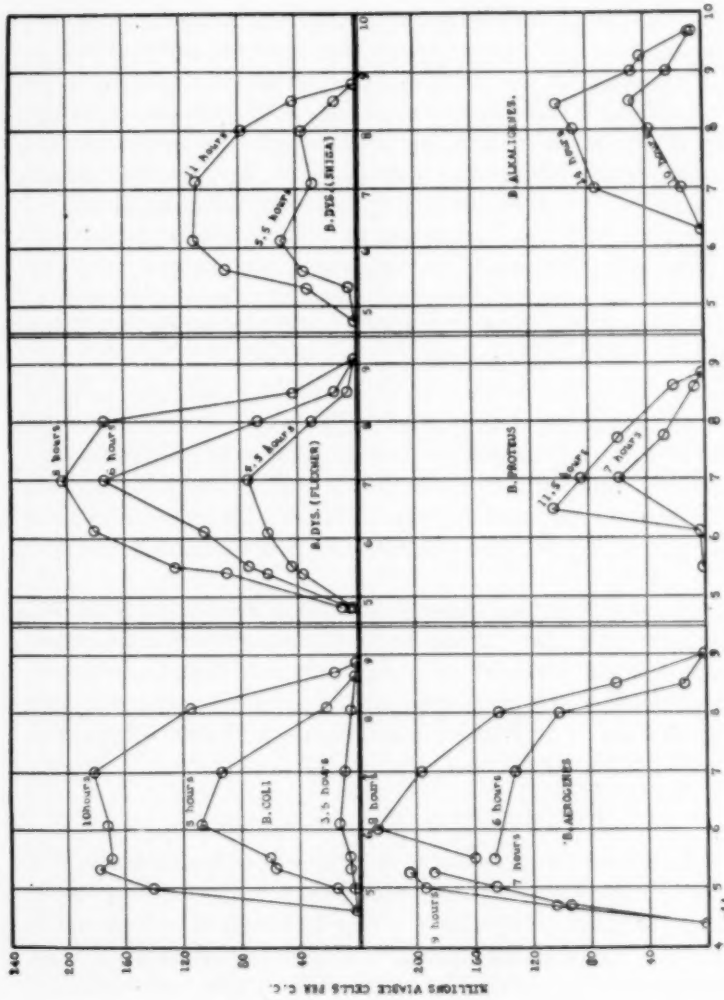

FIG. 3. CURVES SHOWING THE NUMBER OF VIABLE CELLS (ORDINATES) IN CULTURES OF DIFFERENT pH (ABSCISSAE) AT HOURS INDICATED

In other words, *the period of "lag" or "latency" is very distinct and prolonged in the more alkaline media.* It remains to be determined whether or not this indicates that cells transferred from a neutral to an alkaline medium require time for acclimatization.

We cannot give a complete picture of the whole course of the growth curve for our observations in most cases covered but the first ten hours, but in figure 3 are plotted a few of the measurements—sufficient to indicate the influence of pH during the early hours of incubation. Total numbers expressed as millions per cubic centimeter are plotted as ordinates and the pH values of the media as abscissae. Points for the same hour are connected to show, as it were, the rise of the wave of growth with the crest in the optimal zone. The discontinuity in the curves for *B. aerogenes* is due to the fact that two sets of experiments are represented, in one of which the original inoculum was much larger than in the other.

These curves are similar to those published by Shohl and Janney (1917) and indicate somewhat more distinct optima than do the data on maximal rates as illustrated by figure 1; but the reader should be reminded that the period covered is only the first ten hours.

As stated before, the unknown factor or factors may be predominating and may flatten these curves until the hydrogen ion effect is obscured; but even so it appears that the effect of pH is to be found in the period of lag, likewise to some extent within the period of logarithmic increase, and also in the subsequent period.

EXPERIMENTS WITH B. BULGARICUS

We treat these experiments separately in order to emphasize the fact that the media employed were different from those used in the other experiments. This emphasis is necessary because we wish to guard against a too literal interpretation of the comparison we shall make between the conduct of *B. bulgaricus* and *B. coli* at pH 4.5.

Multiplication studies were carried out as described before, but in a medium consisting of the extract of 10 grams dried

brewers' yeast in 1 litre of water containing 1 per cent "Difco" peptone, 0.5 per cent K_2HPO_4, 0.2 per cent succinic acid and 1 per cent glucose. The same medium without the succinic acid but with agar was used in the plates.

Within the short and early period when logarithmic growth occurred the change in pH was not significant, but later considerable changes occurred as the sugar underwent acid fermentation. When data from these later periods are used the pH value observed at the time of the count is given.

B. bulgaricus like other organisms exhibits a remarkably abrupt change in conduct within a narrow range of pH. At pH 4.5 there was a slow but consistently steady decline in numbers during the fifteen hours of observation, while at pH 4.7 we found in one case increases during thirteen hours with a maximal rate of 1.37 generations per hour for the first period of two hours, and in a second case an initial rate of 1.21 and a slower increase in numbers during sixteen hours.

As we have already stated it is perhaps unfair to compare the conduct of *B. bulgaricus* in one medium with the conduct of *B. coli* in another of the same pH; but the fact that *B. bulgaricus* was checked at pH 4.5, when considered beside the fact that *B. coli* cultures can sometimes attain this acidity, suggests that we have here a possible explanation of why it is that under certain conditions found in the manufacture of dairy products *B. coli* gains and retains a supremacy over *B. bulgaricus*. Ordinarily *B. bulgaricus* suppresses *B. coli* for the *fermentative* activity of the former brings a medium well past the point endured by *B. coli*, and the particular organism with which we are now dealing can bring its culture medium to pH 3.9 (Clark, 1916). It is less severely affected at pH 3.9 than is *B. coli* slightly above its own limiting pH. The possible relation of this fact to the experiments of Gratz (1912) is suggestive.

From pH 5.1 to pH 6.7 the generations per hour average 1.6. At 6.7 a period of "lag" becomes evident. At 7.5 the lag became a period of decline five hours in length, after which there was a slow recovery in numbers. This recovery reached a maximal rate of 0.42 between the twelfth and fourteenth hours with a gradual increase in acidity to 7.1.

At pH 8.0 there was a constant decline in numbers for sixteen hours and no visible clouding of the medium at the end of twenty-four hours. Here we may call attention to the fact that several workers in their isolation of *B. bulgaricus* have successfully used media adjusted by the titration method to a zone where phenolphthalein is pink. The pH in these cases must have been as much as 8.9 or more. The discrepancy remains to be ex-

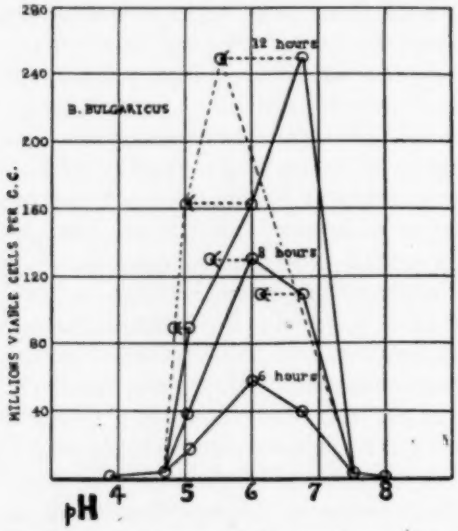

FIG. 4. CURVES SHOWING THE NUMBER OF VIABLE CELLS (ORDINATES) AT THE HOURS INDICATED IN CULTURES OF B. BULGARICUS STARTED AT DIFFERENT pH (ABSCISSAE)

Dotted arrows indicate shift of pH since inoculation

plained, but it may be emphasized that organisms decribed as *B. bulgaricus* which have come under observation in our laboratory are various in type.

A few of the counts made with *B. bulgaricus* cultures are plotted in figure 4. As will be recalled, this organism was studied in a sugar medium in order to secure satisfactory growth for our purposes. The succinic acid as buffer was effective in maintaining the desired pH with insignificant variations in most of

the cultures for six hours or longer; but when fermentation became more vigorous with the increasing numbers of the organism the pH shifted toward the acid side.

It will have occurred to the reader that we have in the data briefly outlined in the preceding pages some evidence that the effects of acidity on growth and on fermentation are independent. A striking evidence of this is to be found in the experiments with *B. bulgaricus*. Cultures of this organism were found to reach pH values consistently near 3.9 (Clark, 1916), yet growth we now find is checked at pH 4.5.

THERMAL DEATH RATES

At extreme reactions we encounter declines in the number of viable cells even at room temperature. It is obvious that we are here approaching the phase of the subject which is classified for convenience under the term thermal death rate. Long ago Pasteur (1879) in writing of the different temperatures required for the "pasteurization" of different materials said:

> It is easy to show that these differences in temperature which are required to secure organic liquids from ultimate change depend exclusively upon the state of the liquids, their nature and above all upon conditions which affect their neutrality whether towards acids or bases.[2]

Hitherto unpublished data obtained some years ago by Clark with the assistance of Mr. Eldridge may be of interest when viewed beside the material presented in this paper.

Cultures of *B. coli* were grown eighteen hours at 30°C. in 1 per cent Witte peptone, 1 per cent glucose broth. Then to aliquot portions of this culture varying quantities of acid were added. The portions were slightly diluted with sterile water to bring each to the same volume, the pH was measured electrometrically and 5 cc. samples were placed in thin-walled vessels in a 45°C. water-bath which was vigorously stirred. In table 3 are given the amounts of hydrochloric, acetic and formic acids added to

[2] It is interesting to note the exact language used by Pasteur in the last phrase.

100 cc. of a culture, the resulting pH, and the time and logarithm of the counts.

TABLE 3

Influence of pH and different acids on the number of viable B. coli surviving a temperature of 45°C. for different lengths of time

N/1 HYDROCHLORIC ACID				N/1 ACETIC ACID				N/1 FORMIC ACID			
Cubic centimeters	pH	Time	Log number	Cubic centimeters	pH	Time	Log number	Cubic centimeters	pH	Time	Log number
		hours				*hours*				*hours*	
0.0	5.16	0	8.55	0.0	5.61	0	8.22	0.0	5.99	0	7.80
		1	7.83			1	8.32			1	7.99
		4	8.02			4	7.97			4	8.22
0.5	4.43	0	8.55	1.5	4.44	0	8.22	1.0	4.03	0	7.80
		1	8.13			1	7.70			1	4.08
		4	7.76			4	6.72			4	2.00
1.5	3.33	0	8.55	7.5	3.79	0	8.22	2.0	3.75	0	7.80
		1	5.45			1	4.24			1	2.00
		4	3.45			4	0.00			4	0.00
2.0	2.79	0	8.55					3.0	3.50	0	7.80
		1	2.60							1	0.00
		4	0.00							4	0.00
2.5	2.37	0	5.55								
		1	0.00								
		4	0.00								

The nature and plan of this series of experiments recall in a general way the work done by Winslow and Lochridge (1906) upon the disinfecting action of certain mineral and organic acids upon colon and typhoid organisms. The essential difference is in the fact that these authors maintained a temperature of 18°C. while the results here reported were observed at 45°C. Winslow and Lochridge noted the marked toxic effect of acetic acid—an effect much greater than that due to the hydrogen ion concentration of the acid. They explained this as being due to the undissociated acid remaining in the solution. It is however not improbable that the free acetate radicle may exert a synergic

effect upon the disinfecting power of the hydrogen ion. A positive determination of such a suggested synergic action may add appreciably to our knowledge of the mechanism of disinfection. Perhaps the difference corresponds to a difference in the permeabilities of the mineral and organic acids.

The conditions of these earlier experiments are obviously such that direct comparison with the present experiments can not be made, but the results indicate the justice of a remark by Clark and Lubs (1917a) based upon this data and the experience of Pasteur: ". . . . in cellular destruction temperature is to be considered as an accelerating *condition* . . . . among the active *agents* concerned the concentration of the hydrogen ions may be of great significance." Buchanan (1917) has recently studied this problem in relation to canning. It is a question of another order whether the coefficients of destruction at higher temperatures become such that there is an *apparent* thermal death *point* useful when considered as such in the pasteurization of milk or other liquids. At the low temperatures of the cheese-curing room, the broader point of view is the more practical. It is also clear that in the practical application of these considerations, as for instance in canning, the variation of the effects observed with different acids in media of the same pH must be kept in mind.

SUMMARY

Although various factors, some of which are evidently quite unknown, may obscure the effect of the hydrogen ion, our preliminary study under the limited set of conditions prevailing indicates that:

1. There is a broad zone of pH within which the rates of growth are quite uniform for those short periods during which the increase of viable cells approaches the logarithmic rate.

2. On the borders of these zones of pH slight change in the pH produces a marked effect upon reproduction.

3. The acid border shifts with the nature of the acid. A distinct difference between the effect of hydrochloric and that of acetic acid was noted.

4. While no distinct optimum reaction was found when only the logarithmic increase was considered, more distinct optima appear when longer periods of growth are observed.

5. The period of "lag" is more prononced in alkaline than in acid media.

6. Evidence is presented which suggests that the effect of pH upon specific fermentative processes, upon reproduction in its several stages and upon rates of death must be kept distinct.

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